

A STUDY OF MARINE BENTHIC ALGAE ALONG A  
NATURAL CARBON DIOXIDE GRADIENT

by

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# **A Study of Marine Benthic Algae Along a Natural Carbon Dioxide Gradient**

Vivienne Johnson

## **Abstract**

Increasing atmospheric CO<sub>2</sub> is causing unprecedented changes in seawater chemistry, yet the uncertainty of the ecological response to these projected changes, termed ‘ocean acidification’, remains considerable at present. To predict the effects of these changes, we need to improve our understanding of the responses of marine primary producers since these drive biogeochemical cycles and determine the structure and function of benthic habitats. The majority of experiments on the effects of ocean acidification on photoautotrophs to date have mainly focused on oceanic microalgae, leaving benthic assemblages largely overlooked. Carbon dioxide vents are providing a means for examining and predicting the impacts of ocean acidification on marine ecosystems. In this thesis a temperate CO<sub>2</sub> volcanic vent gradient was used to investigate the responses of benthic microalgal assemblages (periphyton, epilithic, epipellic, epipsammic and endolithic) and macroalgae (a calcified phaeophyte, crustose coralline algae and turf algae) to increasing *p*CO<sub>2</sub>. The photosynthetic standing crop of microphytobenthic assemblages increased significantly with elevations in CO<sub>2</sub> indicating that the productivity of shallow water habitats may be promoted over the course of this century. Some benthic diatoms appear to benefit in naturally CO<sub>2</sub> enriched environments whilst benthic cyanobacteria in this study appear to be relatively insensitive to the levels of increase predicted for this century. Dramatic shifts in epilithic macroalgae assemblages were observed along the CO<sub>2</sub> gradient and a calcified phaeophyte was revealed as an unexpected ecological winner under ocean acidification scenarios. These observations suggest that benthic algal assemblages have the potential to dramatically alter as CO<sub>2</sub> levels continue to rise; this would have profound consequences for the structure and function of benthic ecosystems.

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### Abbreviations

**CO<sub>2</sub>** - Carbon dioxide  
**H<sub>2</sub>CO<sub>3</sub>** – Carbonic acid  
**CaCO<sub>3</sub>** - Calcium carbonate  
**CO<sub>3</sub><sup>2-</sup>** - Carbonate  
**Ca<sup>2+</sup>** - Calcium  
**HCO<sub>3</sub><sup>-</sup>** - Bicarbonate  
**DIC** - Dissolved inorganic carbon  
**TA** - Total alkalinity  
**CCM** - Carbon concentrating mechanisms  
**Chl *a*** – Chlorophyll *a*  
**CLSM** – Confocal laser scanning microscope  
**SEM** – Scanning electron microscope

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

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- Molecular biology and carbonate chemistry workshop 30/11/09-02/12/09. Zoology Department, Oxford University.
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**Johnson VR**, Russell BD, Fabricius KF, Brownlee C, Hall-Spencer JM (2012b). Temperate and tropical brown macroalgae thrive, despite decalcification, along natural CO<sub>2</sub> gradients. Global Change Biology 18: 2792-2803 (I.F 6.862)

Some of the data within this thesis also contributes in part to the following publications:

Lidbury I, **Johnson VR**, Hall-Spencer JM, Munn CB, Cunliffe M (2012) Community-level response of coastal microbial biofilms to ocean acidification in a natural carbon dioxide vent system. Marine Pollution Bulletin, DOI:10.1016/j.marpolbul.2012.02.011 (I.F 2.503)

Suggett DJ, Hall-Spencer JM, Rodolfo-Metalpa R, Boatman TG, Payton R, Pettay T, **Johnson VR**, Warner ME, Lawson T (2012) Sea anemones may thrive in a high CO<sub>2</sub> world. Global Change Biology. DOI: 10.1111/j.1365-2486.2012.02767.x (I.F 6.862)

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## **Chapter 1: General Introduction**

### **1.1 Introduction**

The effects of increasing atmospheric levels of CO<sub>2</sub> on global climate change, particularly global temperature increases, and the impacts of this on marine and terrestrial ecosystems, have received considerable attention in recent decades. More recently, however, an additional consequence of increasing CO<sub>2</sub> has generated scientific interest; the phenomenon of ocean acidification. Increasing concentrations of anthropogenic CO<sub>2</sub> in the atmosphere has the potential to lower the pH of the oceans. The predicted changes in CO<sub>2</sub> availability may affect marine photoautotroph physiology whilst the associated decrease in the carbonate saturation state of seawater poses a potentially serious threat to calcifying taxa. The issue first gained widespread recognition following a publication by Caldeira & Wickett (2003) and since then ocean acidification has quickly risen to prominence in the marine science community (Gattuso & Hansson 2011). Major scientific questions centre on the potential impacts on marine ecosystems and biogeochemical cycles over the course of the next century.

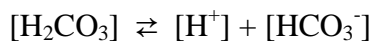
This chapter aims to summarise the current level of understanding concerning responses of marine algae to elevated CO<sub>2</sub>. Marine photoautotrophs are responsible for the ocean's primary productivity which constitutes around 50 % of global primary production (Field et al. 1998). Additionally, marine algae play a crucial role in the export of fixed carbon to the deep ocean, decelerating the accumulation of CO<sub>2</sub> in the oceans and atmosphere, a process known as the 'biological pump' (Ducklow et al. 2001). In light of their ecological and biogeochemical significance it is essential for ocean acidification research to elucidate the effects of increasing CO<sub>2</sub> on marine photoautotrophs.

## 1.2 Ocean Acidification

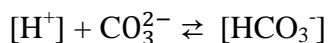
Increasing atmospheric CO<sub>2</sub> is causing unprecedented changes in seawater chemistry (Guinotte & Fabry 2008). Atmospheric CO<sub>2</sub> concentrations are projected to increase by 0.5 % per year throughout the 21<sup>st</sup> century, this rate of change is around 100 times faster than has occurred in the past 650,000 years (Meehl et al. 2007). The current concentration of atmospheric CO<sub>2</sub> is 385 ppm, having increased from pre-industrial levels of 280 ppm, and emissions scenarios predict a continued increase to ~750-1000 ppm by 2100 (IPCC 2007; Feely et al. 2009). The rising atmospheric CO<sub>2</sub> levels drive changes in seawater chemistry and lower pH (Gattuso & Buddemeier 2000). The oceans play a crucial role in the global carbon cycle, forming an important sink for anthropogenic CO<sub>2</sub>. The carbonate equilibrium in seawater is the balance between dissolved inorganic carbon (DIC) species; CO<sub>2(aq)</sub>, HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> ions. Changes in pH control this equilibrium; therefore increasing CO<sub>2</sub> will increase the total DIC and shift the proportion of DIC speciation in seawater, with widespread biological consequences. When CO<sub>2</sub> dissolves in seawater it reacts with water molecules to form carbonic acid:



Carbonic acid then dissociates to bicarbonate and hydrogen ions:



This leads to a lowering of pH and other chemical changes collectively termed as ocean acidification (Calderia & Wickett 2003). The increase in hydrogen ion concentrations causes some carbonate ions to react with hydrogen to become bicarbonate:



Therefore, the dissolution of CO<sub>2</sub> in seawater increases concentrations of hydrogen ions, carbonic acid and bicarbonate whilst decreasing the concentrations of carbonate. The formation and dissolution of carbonate minerals is expressed as follows:

*Mineral formation* ←



→ *Dissolution*

The dissolution of CO<sub>2</sub> in seawater decreases carbonate ion concentrations, shifting the equation to the right, impeding the formation of carbonate minerals and promoting dissolution. This is likely to negatively impact a wide range of calcifying marine biota (Leclercq et al. 2000; Riebesell et al. 2000; Gazeau et al. 2007; Lombardi et al. 2011).

This dissolution of carbonate minerals produces carbonate ions that can react to consume hydrogen ions which in turn counteracts some of the hydrogen generating effects of CO<sub>2</sub> enrichment (Morse et al. 2007). However, as CO<sub>2</sub> is being absorbed so rapidly, it is unlikely that this natural buffering capacity of the ocean surface will be able to prevent a substantial reduction in ocean pH (Raven et al. 2005).

Over the past 200 years the oceans have absorbed about one third of the total human CO<sub>2</sub> emissions (Sabine et al. 2004) resulting in gradual acidification of seawater. Ocean pH has dropped by 0.1 pH units (corresponding to a 30% increase in hydrogen ion concentration) since the Industrial Revolution and, under predicted emission scenarios, is expected to drop another 0.3-0.4 units, from pH 8.2-8.1 to 7.8-7.6, by the end of this century (Caldeira & Wickett 2003; IPCC 2007; Orr et al. 2010). The current rate of CO<sub>2</sub> release into the atmosphere is capable of driving a magnitude of ocean geochemical changes potentially unparalleled in at least ~ 300 My of Earth history (Hönisch et al.



2012). Current anthropogenic trends in ocean acidification already exceed the level of natural variability by up to 30 times on regional scales and are detectable in many areas of the world's ocean (Friedrich et al. 2012).

Near-future acidification is predicted to have dramatic impacts on some marine species with cascading biological consequences for marine ecosystems (Fabry et al. 2008a,b; Hall-Spencer et al. 2008; Doney et al. 2009a,b; Kroeker et al. 2010; Dupont et al. 2010; Abbasi & Abbasi 2011). These studies indicate that variation in the sensitivity of organisms to ocean acidification is likely to disrupt the species balance of communities and influence species interactions which could potentially lead to unforeseen impacts on marine ecosystems.

### **1.3 Response of marine algae to elevated CO<sub>2</sub>**

The effects of increasing atmospheric CO<sub>2</sub> concentrations on the productivity and species composition of terrestrial photoautotrophs has been well documented with extensive laboratory and field research carried out over the last several decades (Wand et al. 1999; Long et al. 2004). Photosynthesis of many terrestrial plants is CO<sub>2</sub> limited so increases in CO<sub>2</sub> are expected to increase rates of photosynthesis on land. Studies have predicted that global plant productivity will increase in response to atmosphere CO<sub>2</sub> enrichment in a range of terrestrial habitats from forests to agro-ecosystems (Saxe et al. 1998; Lee et al. 2001; Fuhrer 2003). In comparison, research into the responses of marine photoautotrophs to CO<sub>2</sub> enrichment is much less advanced, and there are currently no reliable estimates of how global ocean productivity will change in relation to high CO<sub>2</sub>/low pH (Joint et al. 2011).

As CO<sub>2</sub> is a prime substrate for photosynthesis, an increase in  $p\text{CO}_2$  levels may improve growth conditions for some marine algae. Indeed, some oceanic diatoms have been shown to be CO<sub>2</sub> limited under present day seawater concentrations (Riebesell et al. 1993), although these experiments were performed by varying the pH to unrealistic values, making interpretation difficult (Beardall & Giordano 2002). All known algal RUBISCOs (Ribulose-1,5-bisphosphate carboxylase oxygenase), enzymes key to carbon fixation, have competitive carboxylase and oxygenase functions and have relatively low substrate-saturated carboxylase activities on a protein mass basis (Giordano et al. 2005). The relative extent of these two RUBISCO activities depend on the molecular nature (the specificity factor) of the enzyme and concentrations of O<sub>2</sub> and CO<sub>2</sub> at the RUBISCO active site. Different species of photoautotrophs possess different forms of RUBISCO, for example, the form II RUBISCO of dinoflagellates has a lower carboxylation: oxygenation specificity factor compared with the form ID RUBISCO found in diatoms and coccolithophores (Reinfelder et al. 2011). The low concentrations of free CO<sub>2</sub> in seawater under present day atmospheric concentrations do not saturate the carboxylase reaction of RUBISCO which sets a limit for photosynthesis and growth (Badger et al. 1998; Beardall & Raven 2004).

To overcome limitations of an inefficient RUBISCO and maintain high rates of photosynthesis at low concentrations of CO<sub>2</sub>, most marine algae have evolved carbon concentrating mechanisms (CCM; Giordano et al. 2005; Reinfelder et al. 2011). The ubiquitous enzyme carbonic anhydrase (CA) plays a key role in CCM of marine algae (Litchfield & Hood 1964). Experimental evidence points to two types of biophysical CCM; in the first CO<sub>2</sub> is produced by the CA-catalysed dehydration of HCO<sub>3</sub><sup>-</sup> at the cell surface and enters the cell through passive diffusion, in the second model HCO<sub>3</sub><sup>-</sup> is transported across the cell membrane and converted to CO<sub>2</sub> by internal CA (Reinfelder et al. 2011). CCM utilise bicarbonate, which is 140 times more abundant in seawater

than free CO<sub>2</sub> (Raven et al. 1995). Consequently, in contrast to terrestrial systems, CO<sub>2</sub> availability is generally not considered to be limiting to marine photosynthesis (Raven 1997) and marine productivity is therefore generally expected to exhibit relatively little response to increased atmospheric CO<sub>2</sub> (Burkhardt et al. 2001; Israel & Hophy 2002). There may be indirect benefits of increasing concentrations of CO<sub>2</sub> for those species that possess CCM. The operation of CCM require energy expenditure, therefore CCM capacity may be down-regulated by elevated CO<sub>2</sub> allowing algae to reallocate energy for other purposes (Giordano et al. 2005; Hurd et al. 2009).

The carbon acquisition abilities and efficiencies of CCM differ between algae. Different types of CCM are present in almost all algal groups (Giordano et al. 2005). Therefore it is believed that rising CO<sub>2</sub> will affect different species to varying degrees (Hein & Sand-Jensen 1997; Badger et al. 1998; Tortell et al. 2000; Rost et al. 2003; Riebesell 2004; Fu et al. 2008a) potentially resulting in drastic community shifts (Porzio et al. 2011). Furthermore, the availability of CO<sub>2</sub> for photosynthesis in natural habitats is variable, therefore differences in the saturation degree of photosynthesis by CO<sub>2</sub> between algal communities also needs to be considered when modelling their responses to ocean acidification (Mercado & Gordillo 2011). As an additional consequence of CO<sub>2</sub> enrichment, marine photoautotrophs will become exposed to decreasing seawater pH and be influenced by alterations in seawater carbonate chemistry. Therefore a wide range of calcifying macro and microalgae are expected to exhibit strong negative responses to ocean acidification (Riebesell et al. 2000; Martin et al. 2008; Martin & Gattuso 2009).

### 1.3.1 Responses of marine microalgae to elevated CO<sub>2</sub>

The ecological significance of marine microalgae cannot be understated. Marine phytoplankton account for approximately 50% of global primary productivity (Falkowski et al. 1998), representing a major basis of marine food webs. They also underpin a range of important global biogeochemical processes such as nutrient cycling and carbon storage (Sigman & Boyle 2000; Arrigo 2005). The responses of microalgae to ocean acidification could therefore have wide ranging global ramifications. Changes in seawater pH may affect microalgal cells via a number of possible mechanisms. Energy costs are associated with maintaining an internal pH necessary for cell function under changing external pH conditions (Raven & Lucas 1985). The operation of cellular mechanisms involved in pH homeostasis may be affected by seawater chemistry alterations (Taylor et al. 2011). The reaction rate of enzymes is pH-dependent so deviation from optimum pH may impair cellular function (Hinga 2002). For example, pH reductions inhibit the growth of the haptophyceae *Chrysochromulina* sp. (Hama et al. 2012). In addition, primary productivity in both coastal and open ocean environments has the potential to be affected through pH-dependent speciation of nutrients and metals (Huesemann et al. 2002; Millero et al. 2009; Breitbarth et al. 2010; Shi et al. 2010).

Furthermore, pH at the exterior surface of plankton deviates increasingly from ambient seawater as algal metabolic activity and size increases (Flynn et al. 2012). These authors predicted that such deviations would increase with ocean acidification, leading to many plankton species experiencing pH conditions completely outside their historical range. Microcosm experimentation, however, has revealed that some coastal phytoplankton communities are highly resilient to the level of seawater acidification predicted for this century (Nielson et al. 2010). The authors attributed these findings to the large temporal

variations in pH in productive marine ecosystems which may have caused most coastal plankton species to develop a broad tolerance to changing pH. In contrast, coccolithophores are widely considered to be sensitive to reductions in pH as reductions in calcification with increasing  $p\text{CO}_2$  has been widely reported in the literature (Zondervan et al. 2001; Engel et al. 2005; Beaufort et al. 2011).

Most microalgae have evolved CCM which increase the  $\text{CO}_2$  concentration at the active site of RUBISCO (see Beardall & Giordano 2002; Raven & Beardall 2003; Giordano et al. 2005 for detailed reviews). It is believed that these efficient CCM are the main reason why some species tested to date have shown either no change or only small increases in photosynthetic and/or growth rates under elevated  $p\text{CO}_2$  conditions (Hein & Sand-Jensen 1997; Tortell et al. 1997; Burkhardt et al. 2001; Martin & Tortell 2006). A down-regulation of CCM activity, however, has been observed in experiments when microalgae have been grown in high  $\text{CO}_2$  conditions (Beardall & Raven 1981; Beardall 1989; Collins & Bell 2004). Therefore, due to the high metabolic costs of CCM it has been postulated that those species which operate them may still benefit from  $\text{CO}_2$  enrichment since a down regulation of the CCM may allow for optimised energy and resource allocation (Fridlyand et al. 1996; Beardall & Giordano 2002; Rost et al. 2008; Tortell et al. 2008; Trimborn et al. 2009). Compared to low  $\text{CO}_2$  inducible mechanisms, which are better understood, analysis of high- $\text{CO}_2$  responsive mechanisms in microalgae at the molecular level have only just started (Baba & Shiraiwa 2012). Further research focusing on characterising microalgal acclimatisation mechanisms to conditions of high  $\text{CO}_2$  is essential to improve our understanding of their responses to increasing atmospheric  $\text{CO}_2$ .

The efficiency and regulation of inorganic carbon acquisition is known to vary between different groups of microalgae (Tortell et al. 2000; Giordano et al. 2005) which, as a

consequence, may affect the relative fitness of these groups and their sensitivity to increasing CO<sub>2</sub>. Furthermore, ocean acidification is likely to exert varying impacts on plankton physiology as taxon-specific differences in size, metabolic activity and growth rates during blooms produce very different microenvironments around the algae (Flynn et al. 2012). Taxon and species specific differences in CO<sub>2</sub> and pH sensitivity have been widely observed both in the field and laboratory (Hein & Sand-Jensen 1997; Hinga et al. 2002; Tortell et al. 2002; Rost et al. 2003; Kim et al. 2006; Tortell et al. 2008) indicating that ocean acidification will influence microalgal community composition, species succession and competition. The following sections review the existing literature on the effects of high CO<sub>2</sub> on two ecologically important groups of marine microalgae; diatoms and cyanobacteria, as their responses along a CO<sub>2</sub> gradient are investigated in this thesis.

#### *1.3.1.1. Diatoms*

Diatoms are a dominant phytoplankton taxon in the ocean. They contribute nearly 40% of marine organic carbon production (Nelson et al. 1995) and are responsible for one fifth of primary productivity on Earth (Field et al. 1998). They are important marine silicifiers playing a dominant role in the biological carbon pump, transferring CO<sub>2</sub> from the ocean surface to the deep sea (Eppley & Peterson 1979; Buesseler 1998). Due to their siliceous nature, diatoms are not considered to be particularly sensitive to CO<sub>2</sub> (Milligan et al. 2004) therefore, compared with their calcifying counterparts, coccolithophores, fewer studies have focussed on their response to ocean acidification. Bloom forming diatoms are generally not regarded to be CO<sub>2</sub> limited (however see Riebesell et al. 1993 for exception) as they possess a highly regulated and efficient uptake mechanism for both CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> (Burkhardt et al. 2001). Several diatom species have also been shown to be relatively insensitive to changes in pH (Hinga 2002)

and in some experiments diatoms have shown little response to CO<sub>2</sub> enrichment (Tortell et al. 1997; Burkhardt et al. 1999; Tortell et al. 2000; Kim et al. 2006; Crawford et al. 2011). It has therefore been assumed that the effect of CO<sub>2</sub> on photosynthesis and growth in diatoms may be small, especially when compared with other taxa.

More recently, however, it has been argued that CO<sub>2</sub> enrichment may benefit diatoms through down regulation of CCM capacity and the reduced energy costs of carbon fixation (Beardall & Giordano 2002; Rost et al. 2008; Trimborn et al. 2009). This notion helps explain those studies that have observed positive responses of diatoms to CO<sub>2</sub> enrichment (Riebesell et al. 1993; Burkhardt & Riebesell 1997; Burkhardt et al. 1999; Feng et al. 2009; de Kluijver et al. 2010; Sun et al. 2011; Witt et al. 2011; Gao et al. 2012). Hopkinson et al (2011) predicted that a doubling of ambient CO<sub>2</sub> would save around 20 % of the CCM expenditure, decreasing the amount of energy expended on carbon fixation by 3 to 6 % and increasing primary production. This has important ecological ramifications along with the potential to influence biogeochemical cycles and oceanic feedbacks to increasing atmospheric CO<sub>2</sub>.

Variations in CO<sub>2</sub> concentrations can cause shifts in competition, dominance and composition of diatoms in phytoplankton assemblages, attributed to taxon specific differences in CO<sub>2</sub> sensitivity (Tortell et al. 2002, 2008; Kim et al. 2006; Trimborn et al. 2009). When natural phytoplankton assemblages in the Antarctic were subjected to low CO<sub>2</sub> concentrations, diatom abundance substantially decreased, whilst non-siliceous phytoplankton genus, *Phaeocystis*, increased in abundance (Tortell et al. 2002). Field data from another study revealed a CO<sub>2</sub> -dependent species shift in a diatom community with elevated CO<sub>2</sub> conditions stimulating the growth of the larger chain forming *Chaetoceros* spp. (Tortell et al. 2008). Phenotypic variability within diatom populations

may play an important role in determining their responses to elevated CO<sub>2</sub>. Strain-specific growth rates can influence responses, with slow growing cultures responding positively to high CO<sub>2</sub> whilst fast growing cultures show neutral or negative responses (Kremp et al. 2012). This emphasises that responses observed in single strain experiments may not be representative of natural populations under future high CO<sub>2</sub> conditions.

#### *1.3.1.2. Cyanobacteria*

Cyanobacteria are the largest and the most widely distributed group of photosynthetic prokaryotes (Burns et al. 2005) and contribute up to 50 % of fixed carbon in marine systems (Partensky et al. 1999). Diazotrophic cyanobacteria increase the bioavailability of nitrogen by fixing inert atmospheric nitrogen into more reactive forms (ammonia, nitrate, nitrite). Cyanobacteria therefore support a large fraction of primary productivity, particularly in tropical and sub-tropical regions, and play significant roles in the marine nitrogen cycle and global carbon cycle (Gruber & Sarmiento 1997; Copone et al. 2005; Mahaffey et al. 2005). They have a form of RUBSICO that has a very low affinity for CO<sub>2</sub> (Badger et al. 1998; Kaplan & Reinhold 1999) and as a result have developed a highly effective CCM that is thought to be one of the most effective of any photosynthetic organism (Badger & Price 2002). The substantial energetic and metabolic costs associated with this mechanism imply that increasing CO<sub>2</sub> concentrations could prove to be beneficial to cyanobacteria through down regulation of CCM capacity. This idea is supported by data showing that resource allocation between photosynthesis, carbon acquisition and nitrogen fixation in cyanobacteria improves with CO<sub>2</sub> enrichment (Kranz et al. 2009).



The majority of ocean acidification studies on cyanobacteria have focused on the response of bloom forming *Trichodesmium* spp. (Barcelos e Ramos et al. 2007; Hutchins et al. 2007; Levitan et al. 2007; Kranz et al. 2011). These studies report substantial increases in the rates of photosynthesis, N<sub>2</sub> fixation and cell division with CO<sub>2</sub> enrichment. Culture studies of the genus *Crocospaera* have also revealed increases in the rates of nitrogen and carbon fixation, as well as growth, with elevated CO<sub>2</sub>, although to a lesser extent than *Trichodesmium* spp. (Fu et al. 2008a). Lu et al. (2006) also recorded positive effects of CO<sub>2</sub> enrichment on the genus *Synechococcus*. These experiments indicate that increasing levels of CO<sub>2</sub> could significantly alter nitrogen inputs to the ocean and profoundly affect global biogeochemical cycles (Hutchins et al. 2009).

It appears that the cyanobacterial response to ocean acidification can be both species and strain specific. In contrast to *Trichodesmium* spp., the bloom forming species *Nodularia spumigena* reacted negatively to seawater acidification with reduced cell division and nitrogen fixing rates (Czerny et al. 2009). When exposed to high CO<sub>2</sub> concentrations the genus *Synechococcus* spp. responded with higher rates of growth and photosynthesis while *Prochlorococcus* spp. was relatively unaffected (Fu et al. 2007). Different strains of *Synechococcus*, have all been found to respond positively to CO<sub>2</sub> enrichment but to varying degrees (Lu et al. 2006). The contrasting responses of various species of cyanobacteria to CO<sub>2</sub> is thought to be due to differences in the ecological strategies between heterocystous (contain specialised nitrogen-fixing cells) species (*Nodularia*) and non-heterocystous (*Trichodesmium*) species (Czerny et al. 2009).

### **1.3.2. Responses of marine macroalgae to elevated CO<sub>2</sub>**

Macroalgae are an important functional component of near-shore benthic environments (Smith 1981; Charpy-Roubaud & Sournia 1990; Gattuso et al. 1998), yet research into understanding the impacts of elevated CO<sub>2</sub> on these communities is relatively scant. Photosynthesis in most of the intertidal macroalgal species studied to date has been shown to be CO<sub>2</sub> saturated (Surif & Raven 1989; Beer 1994), which has been attributed to the possession of CCM. Therefore, it was previously believed that seaweed performance would not be enhanced with increasing CO<sub>2</sub> (Beardall et al. 1998). However, it is now thought that down-regulation of macroalgal CCM may be beneficial to some species (Hurd et al. 2009). In addition, there are species that rely on the passive uptake of dissolved CO<sub>2</sub> as a carbon source for photosynthesis, and are consequently CO<sub>2</sub> limited under present conditions (Maberly 1990; Raven et al. 2002a,b). These seaweeds are likely to directly benefit from increasing rates of CO<sub>2</sub> diffusion under ocean acidification; many sub-tidal species, for example, are thought to be CO<sub>2</sub> limited under present day conditions (Holbrook et al. 1988; Johnston et al. 1992).

There is, therefore, potential for shifts in macroalgal communities from carbon-saturated to presently carbon-limited species as  $p\text{CO}_2$  levels continue to rise in the surface ocean. Additionally, the associated decrease in carbonate ion availability is likely to have severe consequences for calcifying macroalgae, whilst enhancing competitive abilities of non-calcified species. Elevations in CO<sub>2</sub> therefore have the potential to alter the competitive outcomes among non-calcified species, with and without CCMs, and those that calcify, profoundly affecting future macroalgal community structure (Hepburn et al. 2011; Hofmann et al. submitted). The following sections review the published effects of

elevated CO<sub>2</sub> concentration on both calcareous and non-calcareous macroalga taxa as their responses along a CO<sub>2</sub> gradient were also investigated in this PhD thesis.

#### *1.3.2.1. Calcareous macroalgae*

The ecological and biogeochemical importance of benthic calcifying macroalgae is well documented. The order Corallinales contains crustose coralline algae which are significant framework builders and play an important stabilising role on coral reefs (Littler & Littler 1984). Crustose coralline algae also provide an important hard substratum for coral larvae and are known to induce mesoplankton settlement (Heyward & Negri 1999; Harrington et al. 2004). Erect, articulated taxa such as *Halimeda* and *Amphiroa* are important contributors to the production of marine sediments (Drew 1983; Rees et al. 2007), consolidate reef accretion by infilling gaps in the reef matrix with their sediments (Drew & Abel 1985) and provide important nursery habitats for many reef fish and invertebrates (Beck et al. 2003). Epibiotic calcareous algae are also important in seagrass meadows where they are major contributors to CO<sub>2</sub> fluxes in the carbon cycle, a consequence of their high carbonate production and dissolution rates (Barro'n et al. 2006).

The effect of reduced pH on calcified macroalgae has been well researched over the last decade producing a substantial body of evidence highlighting the negative effects on crustose coralline algae (Leclercq et al. 2000, 2002; Anthony et al. 2008; Jokiel et al. 2008; Kuffner et al. 2008; Martin et al. 2008; Andersson et al. 2009; Martin & Gattuso 2009; Russell et al. 2009; Semesi et al. 2009; Büdenbender et al. 2011; Fabricius et al. 2011; Doropoulos et al. 2012), erect articulated genera (Borowitzka & Larkum 1976; Borowitzka 1981; Gao et al. 1993a; de Beer & Larkum 2001; Langdon et al. 2000; Robbins et al. 2009; Couto et al. 2010; Price et al. 2011; Porzio et al. 2011; Sinutok et

al. 2011) and free-living forms, maërl (Burdett et al. 2012) The majority of these studies have shown that the elevation in CO<sub>2</sub> predicted to occur over the course of this century will lead to decreased calcification, growth and abundance of calcified macroalgae.

Corallinaceae are among the most sensitive calcifying organisms to ocean acidification. Their skeletal composition is in the form of high-magnesium calcite, the most soluble form of CaCO<sub>3</sub> (Milliman 1974) and, as a result have not been found in naturally acidified water where other calcifiers survive (Hall-Spencer et al. 2008; Martin et al. 2008). There is concern that increasing CO<sub>2</sub> concentrations may ultimately lead to the dissolution of the calcified skeletons of coralline algae (Orr et al. 2005; Kleypas et al. 2006; Martin & Gattuso 2009; Manzello et al. 2008). It has been predicted that when levels reach approximately 450 ppm (due to occur by 2030-2040 at current emission rates) coralline algal calcification will become completely inhibited (Kelypass & Langdon 2006; Yates & Halley 2006; Anthony et al. 2008; Veron et al. 2009). In addition to hindering calcification, ocean acidification has also been found to interfere with the recruitment stages of crustose coralline algae (Jokiel et al. 2008; Kuffner et al. 2008). For example, reductions in pH disrupt intimate coral larvae-algal settlement interactions; larvae behaviour has been shown to switch from settlement on high preference crustose corraline algae to a low preference substrata in low pH, resulting in higher rates of post settlement mortality (Doropoulos et al. 2012) It is apparent from the literature to date that the impacts of ocean acidification on coralline algae are likely to be profound with major biodiversity and biogeochemical consequences in both the benthic temperate and tropical communities dominated by these algae.

General predictions concerning the biological consequences of ocean acidification for calcified macroalgae are, however, difficult to make as individual species, even within a genus, can vary widely in their responses to elevated CO<sub>2</sub> (Price et al. 2011). Studies

investigating the photosynthetic response of calcified macroalgae to increasing CO<sub>2</sub> concentrations have also revealed contrasting results. Photosynthetic performance and/or growth rates have been found to both increase (Reiskind et al. 1988; Gao et al. 1993a; Semesi et al. 2009), decrease (Langdon et al. 2000; Tribollet et al. 2006b; Price et al. 2011; Sinutok et al. 2011) or to show insensitivity (Leclercq et al. 2002) to CO<sub>2</sub> enrichment. Meta-analysis (Kroeker et al. 2010) revealed that the responses of calcified algae to ocean acidification are highly variable. A recent study has shown that ocean acidification could have potentially profound consequences for the functional diversity of coralline algae (Doropoulos et al. 2012). It suggests that the most sensitive taxa of crustose coralline algae to elevated CO<sub>2</sub> are early successional species characterised by rapid growth and thin thalli, whilst taxa that are late successional with thick crusts (> 500 µm) may be more resistant.

Despite the bleak prognosis for calcified macroalgae due to ocean acidification, some *in situ* observations show that some calcified species (*Hydrolithon cruciatum* and *Peyssonnelia squamaria*) can grow successfully at mean pH 7.8 (Porzio et al. 2011). The responses of *Halimeda* spp. to ocean acidification have been shown to be species specific with one species, *Halimeda taenicola* demonstrating potential for acclimatisation to low pH by lowering the amount of CaCO<sub>3</sub> required in the thallus (Price et al. 2011). Additionally, in one laboratory study, the coralline red alga, *Neogoniolithon* sp., and the calcareous green alga *Halimeda incrassata* both exhibited increased calcification due to moderately elevated CO<sub>2</sub> (Ries et al. 2009). In contrast to previous predictions (Kelypas & Langdon 2006; Yates & Halley 2006; Anthony et al. 2008; Veron et al. 2009) net calcification actually increased under intermediate levels of pCO<sub>2</sub> (606 and 903 ppm) and only declined at extremely elevated levels (2856 ppm). These results, however, may have been confounded by the unappreciated effects of

nutrient addition that commonly occurs when heating temperate seawater for the maintenance of tropical species (Price et al. 2011). Price et al. (2011) suggest that the release from nutrient limitation may have enhanced *Halimeda* productivity, enabling them to photosynthesise in experimental aquaria at rates greater than those possible under natural, oligotrophic reefs, thereby dampening the ability to detect the potential effects of ocean acidification (until pH is lowered enough to produce dissolution effects).

It is apparent that we are at an early stage of fully understanding the implications of increasing dissolved CO<sub>2</sub> concentrations on photosynthesis and calcification in benthic calcified macroalgae. This is due to the conflicting photophysiological responses documented in the literature, our lack of understanding of the complex underlying physiological mechanisms, a heavy bias towards researching rhodophytes and the fact that the interpretation of some responses may be potentially constrained by the nature of ‘shock’ short-term laboratory/mesocosm experiments. With the exception of a few studies/species (Ries et al. 2009; Porzio et al. 2011), it is apparent from the research to date that calcareous macroalgae are highly vulnerable to ocean acidification (Koch et al. 2012). However, since some calcifiers can form their CaCO<sub>3</sub> structures from CO<sub>2</sub> and/or HCO<sub>3</sub><sup>-</sup> and can modify local carbonate chemistry, this may enable them to successfully form calcareous structures despite ocean acidification (Roleda et al. 2012). This, however, should not undermine the serious dissolution threat facing many calcifying algae associated with the levels of ocean acidification predicted for the end of this century (Martin & Gattuso 2009; Büdenbender et al. 2011).

### 1.3.2.2. Non-calcareous macroalgae

A few studies have shown that some species of non-calcareous macroalgae may benefit from ocean acidification as experiments show that rates of growth and photosynthesis have been observed to increase with a rise in CO<sub>2</sub> levels above the present atmospheric levels (Gao et al. 1991, 1993b, Kübler et al. 1999; Connell & Russell 2010; Russell et al. 2011a). The rhodophyte *Lomentaria articulata*, for example, relies exclusively on CO<sub>2</sub> entry by passive diffusion. In response to a doubling of CO<sub>2</sub> concentrations, its growth rate increased by 52 % due to the reduction in the effects of slow CO<sub>2</sub> diffusion rates (Kübler et al. 1999). Like microalgae however, the majority of macroalgae possess CCM which may imply they will show little response to increasing CO<sub>2</sub>. This is supported by previous laboratory manipulations which found, in contrast to the above studies, that growth and photosynthesis in a variety of Mediterranean species of chlorophyte, rhodophyte and phaeophyte were not significantly affected by elevated CO<sub>2</sub> (Israel & Hophy 2002). However, diminished CCM activity can occur when macroalgae are grown under high CO<sub>2</sub> conditions (Raven & Johnston 1991; Magnusson et al. 1996) and therefore down-regulation of CCM may also benefit macroalgal growth through improved energy reallocation (Hurd et al. 2009). Furthermore, non-photosynthetic enhancement of growth by high CO<sub>2</sub> has been reported for *Ulva* sp. (Gordillo et al. 2001). Elevated CO<sub>2</sub> increased algal growth through enhancement of nitrogen assimilation, therefore the effect of CO<sub>2</sub> on algal metabolism may go further than simply acting as a substrate for photosynthesis.

*In situ* experiments investigating macroalgal communities along natural gradients of pH/ CO<sub>2</sub> at volcanic CO<sub>2</sub> vent sites are beginning to reveal important information about the changes in macroalgal community structure that may occur with ocean acidification (Hall-Spencer et al. 2008; Porzio et al. 2011). In a study of 101 macroalgal species it

was discovered that the vast majority were able to tolerate and grow in elevated  $p\text{CO}_2$  levels and mean pH 7.8 (Porzio et al. 2011). Whilst the cover of coralline algae has been found to decrease from > 60% outside vents to zero within it, fleshy benthic macroalgae (such as *Caulerpa*, *Cladophora*, *Asparagopsis*, *Dictyota* and *Sargassum* spp.) increased significantly from near zero to > 60% cover (Hall-Spencer et al. 2008). Similar changes have also been reported across a tropical  $\text{CO}_2$  vent gradient (Fabricius et al. 2011). One concern is that ocean acidification may benefit certain highly invasive non-native species of macroalgae with the ability to cause major changes in the structure and function of coastal ecosystems around the world (e.g. Walker & Kendrick 1998; Piazzini et al. 2001).

Increased  $\text{CO}_2$  may also increase the recruitment and accelerate the expansion of filamentous turf algae, particularly when it is elevated in combination with temperature and nutrients (Diaz-Pulido et al. 2007; Russell et al. 2009; Connell & Russell 2010; Russell et al. 2011a). An expansion of these typically ephemeral assemblages may perturb benthic ecosystems and contribute to ecological phase shifts (Russell et al. 2009; Connell & Russell 2010). Coral reefs, in particular are under threat from expanding macroalgal populations; some data indicate that ocean acidification will shift the competitive interaction between corals and macroalgae in favour of the latter (Diaz-Pulido et al. 2011). Macroalgae play critical roles in reef degradation, inducing ecological phase shifts (McManus & Polsenberg 2004) and reducing coral growth and recruitment (Tanner 1995; Lirman 2001), therefore any positive effect of ocean acidification on the growth of macroalgae may compromise the already fragile state of coral reefs.



Based on the literature to date, the majority of non-calcareous macroalgae appear to be relatively resilient to the effects of ocean acidification, when compared with calcified taxa and may dominate in a higher CO<sub>2</sub> ocean (Koch et al. 2012). Many species have exhibited tolerance to long term reductions in pH (Porzio et al. 2011), even at the microscopic stages of the reproductive life cycle (Roleda et al. 2011), and some have been shown to benefit from elevations in CO<sub>2</sub> (Kübler et al. 1999). Ocean acidification however, is predicted to lead to significant structural and functional changes in coastal macroalgal communities in both tropical (Diaz-Pulido et al. 2007, 2011) and temperate regions (Russell et al. 2009, 2011a; Hepburn et al. 2011; Porzio et al. 2011) with significant ecological consequences.

#### **1.4 Interactive impacts of multiple environmental stressors**

Ocean acidification is not proceeding in isolation; interactive effects of elevated CO<sub>2</sub> with other changing environmental conditions may occur, complicating the prediction of overall ecosystem responses. There has been a recent increase in the number of studies investigating the effects of elevated CO<sub>2</sub> on marine algae whilst incorporating the modulation of other environmental variables such as temperature (Hare et al. 2007; Anthony et al. 2008; Martin & Gattuso 2009; Connell & Russell 2010; Sinutok et al. 2011), nutrients (Russell et al. 2009; Lefebvre et al. 2012) and light (Gao & Zheng 2010; Hepburn et al. 2011; Gao et al. 2012; Hoogstraten et al. 2012). They have revealed that elevated CO<sub>2</sub> can act additively or synergistically with these variables which may have a larger impact on the algae than that of CO<sub>2</sub> alone. For example, results have indicated that CO<sub>2</sub> can act synergistically with seawater warming to lower thermal bleaching tolerances and decrease net calcification in crustose coralline algae (Anthony et al. 2008; Martin & Gattuso 2009). There are concerns that this will

accelerate the deterioration of coral reef habitats (Veron et al. 2009). Reductions in coccolithophore calcification have also been observed as a result of the combined effects of elevated CO<sub>2</sub> and temperature (Feng et al. 2009). Alternatively, temperature and CO<sub>2</sub> can form positive synergies for some algae. Increases in the rate of photosynthesis in the marine cyanobacteria *Synechococcus* were found to be far greater in warm, high CO<sub>2</sub> conditions than the sum of the responses to each factor individually (Fu et al. 2007). Furthermore, combined increases of nutrients and CO<sub>2</sub> can facilitate recruitment of turf algae resulting in them occupying 70 % more space in mesocosms at the expense of calcifying algae (Russell et al. 2009)

Given the predicted climate change scenarios of rising ocean temperatures and ocean acidification (IPCC 2007; Houghton 2009) it is becoming increasingly important to incorporate a variety of environmental variables in ocean acidification studies. Global warming, for example, has the potential to increase thermal stratification in the upper ocean consequently reducing the upwelling of nutrients and thereby limiting the growth and productivity of phytoplankton populations (Behrenfeld et al. 2006). This, in turn, may affect the responses of algae to CO<sub>2</sub> enrichment. Increased surface stratification and decreases in upper-mixed layer depths may also expose phytoplankton to higher light intensities (Boyd et al. 2010). Increased exposure to light has been found to harm primary producers to a greater extent with progressing ocean acidification (Gao et al. 2009; Chen & Gao 2011). In a recent study of South China Sea phytoplankton, whilst elevated levels of CO<sub>2</sub> alone promoted the growth of oceanic diatoms, in combination with increases in surface irradiance, high CO<sub>2</sub> caused a community shift away from diatoms, the dominant phytoplankton group (Gao et al. 2012). In addition, it is likely that the responses of macroalgae to elevated CO<sub>2</sub> will differ between low and high light environments (Hepburn et al. 2011). We are however still at an early stage of

understanding these complex multiple interactions and how they will affect photoautotrophic communities and biogeochemical cycles in our rapidly changing oceans. Future studies need to adopt a multi-factorial experimental approach in order to incorporate the effect of the interactive variables that may enhance and/or decrease the effects of ocean acidification.

## **1.5 Review of Experimental Approaches**

### *1.5.1 Laboratory experimentation and associated limitations*

To date most ocean acidification studies have been experimental laboratory studies, typically conducted in aquaria and small mesocosms. Whilst these types of approaches provide valuable information on global ecological problems (Benton et al. 2007) they are generally only short term (days/weeks) and rapid perturbation experiments focused on single species. In many cases these short term experiments are thought to provoke stress responses (Wood et al. 2008) and produce results which overestimate the impacts of acidification on marine organisms (Hendriks et al. 2010). They are also not temporally representative of the decadal time scales associated with ocean acidification and therefore are unable to account for the contemporary evolution of microalgae (Lohbeck et al. 2012). In addition, many of the experiments studying phytoplankton have relied on manipulating organisms obtained from culture collections which tend not to reflect natural conditions.

With regards to batch cultures of phytoplankton, which are usually maintained at high cell densities, the control of  $p\text{CO}_2/\text{pH}$  is challenged further as the growth of the cells continuously changes the concentration of the inorganic carbon species (Rost et al.

2008). Moreover, most long-term culture facilities do not control pH so in stock cultures phytoplankton may have been growing at pH values that deviate significantly from normal seawater values, often for many decades. This may have resulted in existing culture collections unwittingly selecting for organisms able to grow at high or low pH conditions or for organisms less sensitive to rapid temporal pH variations (Joint et al. 2011).

Perturbation experiments investigating the responses of marine autotrophs to ocean acidification have often yielded contradictory results, even for the same species. This has sometimes been attributed to differences in experimental design (Ratti et al. 2007; Iglesias-Rodriguez et al. 2008; Marubini et al. 2008; Hurd et al. 2009), particularly with regards to the control of seawater carbonate chemistry in laboratory conditions.

However, others argue that whilst the magnitude of the responses may vary, differences in the approaches used to control carbonate chemistry cannot explain contrasting biological responses between these experiments (Gattuso & Lavigne 2009). Furthermore, other, more recent experimental evaluations have discovered that method of acidification and experimental conditions have little influence on some microalgal responses to ocean acidification (Ridgwell et al. 2009; Shi et al. 2009; Findlay et al. 2011), however see Langer & Bode (2011).

A further major limitation to the majority of laboratory studies is that they have assessed organisms in isolation so therefore do not reflect the dynamics of natural marine communities and ecosystems. Single species experiments may not translate into accurate predictions of future ecological change (Russell et al. 2011b). There has been a recent increase in the development of mesocosm experiments (involving outdoor or semi-enclosed tanks) to stimulate natural conditions as closely as possible whilst manipulating  $p\text{CO}_2$  concentrations (e.g. Leclercq et al. 2002; Tortell et al. 2002; Engel

et al. 2005; Jokiel et al. 2008; Andersson et al. 2009; Hicks et al. 2011). These experiments however have been hindered by the difficulties of simulating ocean acidification conditions *in situ* for periods of sufficient length to impact communities of interacting organisms. Furthermore, laboratory/mesocosm perturbation experiments traditionally, have not been designed to encompass the natural variation in the carbonate system experienced in coastal ecosystems, which may have a large impact on the development of resilience in marine populations (Hoffmann et al. 2011). However, an important new experimental approach, the Coral-Proto Free Ocean Enrichment System (CP-FOCE) has recently been developed and tested on the Great Barrier Reef (Kline et al. 2012). Using feedback controls, this *in situ* carbon dioxide perturbation approach maintains experimental pH as an offset from environmental pH thereby incorporating natural diel and seasonal pH cycles. This is an important step forward in the development of CO<sub>2</sub> manipulation experiments that more closely reflect natural conditions.

#### *1.5.2 The use of naturally CO<sub>2</sub> enriched sites in ocean acidification studies and associated limitations*

Field studies at naturally CO<sub>2</sub> enriched sites provide a novel approach for the study of ocean acidification. The utilisation of these natural analogue sites, as a complementary approach to the prevalent *ex situ* investigations, is gaining increasing acceptance in the field of ocean acidification research. This has included the use of hydrothermal vents (Couto et al. 2009; Tunnicliffe et al. 2009; Vizzini et al. 2010; Bianchi et al. 2011), cool water CO<sub>2</sub> vents (Hall-Spencer et al. 2008; Martin et al. 2008; Dias et al. 2010), low-pH coastal springs (Crook et al. 2012) and CO<sub>2</sub> upwelling areas with higher than normal CO<sub>2</sub> concentrations (Manzello et al. 2008; Thomsen et al. 2010). Experiments in naturally acidified environments capture many ecosystem interactions over long time

scales, thereby providing crucial information on the effects of ocean acidification on trophic and competitive interactions as well as the potential for adaption (Gazeau et al. 2011).

Some marine shallow water CO<sub>2</sub> vents are found at ambient seawater temperature and lack toxic sulphur compounds. These are particularly abundant in the Mediterranean and can exist for hundreds of years (Dando et al. 1999). They can therefore be used as underwater laboratories to assess the long term impacts of low pH on entire marine ecosystems. Researchers have begun studies of the ecological changes occurring along natural gradients of CO<sub>2</sub>/pH yielding new and diverse information on the effects of ocean acidification such as where ecological tipping points occur due to rising *p*CO<sub>2</sub> levels (Hall-Spencer et al. 2008; Fabricius et al. 2011; Porzio et al. 2011), impacts of low pH on calcified macroalgae (Martin et al. 2008; Johnson et al. 2012b), down regulation of seagrass phenolics as CO<sub>2</sub> levels increase (Arnold et al. 2012), impacts of high *p*CO<sub>2</sub> on invertebrate settlement, physiology and ecology (Cigliano et al. 2010; Kroeker et al. 2011; Lombardi et al. 2011; Suggett et al. 2012), microbial community dynamics (Lidbury et al. 2012; Meron et al. 2012), microbial ammonia oxidation (Kitidis et al. 2011) and the synergistic effects of increased temperature and high CO<sub>2</sub> (Rodolfo-Metalpa et al. 2010, 2011).

Despite their advantages, shallow water vent systems also have some limitations which are important to consider when using them as an approach to study the impacts of future ocean acidification scenarios. These include abnormal pH variability, recruitment of organisms from unaffected areas, limited spatial replication and the inability to manipulate experimental conditions and control potential confounding factors. These drawbacks are discussed in more detail in Chapter 2.

## 1.6 Summary and Thesis Aims

Ocean acidification is a rapidly emerging field of research (Gattuso & Hansson 2011) and the responses of marine algae to elevated CO<sub>2</sub> are highly variable and complex. It is apparent that there will be non-uniform responses to increasing *p*CO<sub>2</sub> among algal taxa due to differences in carbon acquisition mechanisms (e.g. CCM vs diffusive CO<sub>2</sub> entry and taxa-specific differences in CCM efficiencies) and whether or not they calcify. The known differences in algal response to increased CO<sub>2</sub> levels indicates that changes in the carbonate chemistry of the oceans will have significant effects on the taxonomic composition of pelagic and benthic photoautotrophic communities, with potentially profound ecological and biogeochemical implications. Our current understanding of the impacts of elevated CO<sub>2</sub> on marine algae, however, is relatively limited as it is mostly derived from simplified, artificial experimental set-ups and is hindered by contradictory results in the existing literature. An *in situ*, mensurative approach along natural CO<sub>2</sub> gradients can provide insights that are complimentary in scope and scale to the prevalent *ex-situ* approaches, with the added advantages of incorporating ecosystem interactions and reflecting long term responses.

This thesis describes experiments that utilise a volcanic CO<sub>2</sub> gradient in Sicily to investigate the responses of a variety of marine benthic algae assemblages to *in situ* CO<sub>2</sub> enrichment. Despite their ecological significance, benthic microalgae have been relatively overlooked in ocean acidification studies. We may expect their responses to differ from oceanic microalgal assemblages due to the differences that exist between their physical environments. This thesis therefore focuses on the effects of elevated CO<sub>2</sub> on assemblages of benthic microalgae (diatoms, cyanobacteria) sampled across a variety of different habitats, including: artificial substrata (periphytic assemblages), natural rock surfaces (epilithic biofilms), sandy sediment surfaces (epipsammic and epipelagic

assemblages) and within CaCO<sub>3</sub> substrata (endolithic biofilms). It aims to determine the potential changes in the abundance and composition of these microphytobenthic assemblages with CO<sub>2</sub> enrichment. Changes in the composition of macroalgal communities were also investigated in order to predict the responses of two functionally important algal components of shallow water benthic habitats, crustose coralline algae and turf algae, to ocean acidification. The latter part of this study presents the findings from a study of the common, calcified phaeophyte, *Padina pavonica*. Whilst calcified rhodophytes and chlorophytes have been extensively studied, this thesis provides the first assessment of the effects of ocean acidification on calcified phaeophytes which differ from rhodophytes and chlorophytes in their calcification properties. The occurrence of this genus along a CO<sub>2</sub> gradient provided an opportunity to study its ecological and physiological responses to long-term elevations in CO<sub>2</sub> within natural settings.



## **Chapter 2:** Introduction to Vulcano Island volcanic CO<sub>2</sub> vent system.

Volcanic vents can be used as natural laboratories to assess the impacts of long term exposure to increasing CO<sub>2</sub> levels and lowering carbonate saturation states on marine ecosystems. This chapter describes a shallow, ambient water temperature, volcanic CO<sub>2</sub> gradient off Vulcano Island in the South Tyrrhenian Sea (Italy). Various physical and chemical parameters were monitored across this system over a two and a half year period. The carbonate chemistry profile of this gradient was determined and is presented here for reference in subsequent chapters. The use of this natural CO<sub>2</sub> gradient for investigating and predicting the responses of marine benthic ecosystems to ocean acidification is also discussed.

The data from this chapter contributes to the following published papers:

**Johnson VR**, Brownlee, C, Rickaby REM, Graziano M, Milazzo M & Hall-Spencer, JM (2012a) Responses of marine benthic microalgae to elevated CO<sub>2</sub>. *Marine Biology* DOI: 10.1007/s00227-011-1840-2

**Johnson VR**, Russell BD, Fabricius KF, Brownlee C, Hall-Spencer JM (2012b) Temperate and tropical brown macroalgae thrive, despite decalcification, along natural CO<sub>2</sub> gradients. *Global Change Biology* 18: 2792-2803

Lidbury I, **Johnson VR**, Hall-Spencer JM, Munn CB, Cunliffe M (2012) Community-level response of coastal microbial biofilms to ocean acidification in a natural carbon dioxide vent system. *Marine Pollution Bulletin* 64: 1063-1066

Suggett DJ, Hall-Spencer JM, Rodolfo-Metalpa R, Boatman TG, Payton R, Pettay T, **Johnson VR**, Warner ME, Lawson T (2012) Sea anemones may thrive in a high CO<sub>2</sub> world. *Global Change Biology* DOI: 10.1111/j.1365-2486.2012.02767.x

## 2.1 Introduction

Investigating marine environments naturally exposed to high levels of CO<sub>2</sub> is a new approach to understanding the direct and indirect impacts of ocean acidification at the ecosystem level (Barry et al. 2011). It provides unique opportunities to research the physiological responses and adaptations of marine biota to long term ocean acidification. These systems are particularly useful as they affect many organisms throughout their life history, incorporating multi-species interactions and ecosystem feedback effects which are difficult to replicate in laboratory experiments. Additionally, experiments in natural habitats have the advantage of including a large, dynamic and diverse pool of microbial species present in the ocean which laboratory experiments are unable to mimic (Meron et al. 2012). Volcanic CO<sub>2</sub> venting sites were first utilised as a tool for studying the impacts of ocean acidification on benthic marine environments in 2008 (Hall-Spencer et al.). Bubbling, subterranean sources of CO<sub>2</sub> in the shallow coastal zone produce natural pH gradients along which ecological changes can be examined.

Shallow water CO<sub>2</sub> vents at ambient seawater temperature are particularly abundant in the Mediterranean, where they can persist for hundreds of years (Dando et al. 1999).

Crucially, some lack the sulphur and other toxic compounds that characterise the majority of Mediterranean vents (Dando et al. 1999) therefore the biological responses recorded at these sites cannot be attributed to the presence of potentially toxic compounds. One of the most studied CO<sub>2</sub> vent systems in the field of ocean acidification research is off the island of Ischia, Gulf of Naples, Italy (Hall-Spencer et al. 2008; Martin et al. 2008; Cigliano et al. 2010; Dias et al. 2010; Rodolfo-Metalpa et al. 2010, 2011; Kerrison et al. 2011; Kroeker et al. 2011, 2012; Porzio et al. 2011).

More recently, however, additional CO<sub>2</sub> gradients are beginning to be used to further our understanding of the effects of increasing CO<sub>2</sub> levels on marine life in other

temperate regions (Johnson et al. 2012a; Lidbury et al. 2012; Graziano et al. in review), and in the tropics (Fabricius et al. 2011; Johnson et al. 2012b; Uthicke & Fabricius 2012), in order to better constrain global scale predictions of the impacts of ocean acidification on marine biota.

The CO<sub>2</sub> vent system which forms the main focus of this PhD thesis occurs along a ~300 m stretch of rocky coast off Vulcano Island (38°25'08.52 N, 14°57'39.13 E), approximately 18 miles off the coast of NE Sicily (Figs. 2.1 & 2.2). Vulcano is one of eight volcanic islands in the Aeolian archipelago. There are several submarine volcanic CO<sub>2</sub> vents off Vulcano, the main ones are concentrated in the Baia di Levante (Capaccioni et al. 2001). The Baia di Levante has recently been described as a “natural laboratory” where almost all of the biogeochemical processes related to ocean acidification can be studied (Boatta et al. in review). The gas composition at these vents consists of > 99 % CO<sub>2</sub> (Baubron et al. 1990).

The Baia di Levante is a micro-tidal region where vent activity (at ambient temperatures) acidifies the seawater producing a pH gradient in shallow water (< 3 m) ranging from pH~ 8.2 to ~ 5.6, running parallel to the coast (Fig 2.3 a & b; Boatta et al. in review). The lowest pH levels (~5.5-5.6) are found at the main CO<sub>2</sub> gas vent (Fig. 2.1, 2.2 & 2.3), these are relatively barren areas, although thermophilic biofilms which can tolerate the low pH are abundant (Appendix I a & b). Hydrogen sulphide, which is potentially toxic to cellular respiration, has been recorded at the vent sources, brought in by hydrothermal fluids (Sedwick & Stüben 1996). However, oxidation of this to non-toxic sulphate in the oxygen rich environment of the bay, alongside low sulphide concentrations (< 50 µMol kg<sup>-1</sup>), suggests that only a small proportion enters or remains in the aquatic phase at > 20 m distances from the main vent area (Boatta et al. in

review). The study sites used in this thesis were at distances > 20 m from the main vent area, at ambient temperature.

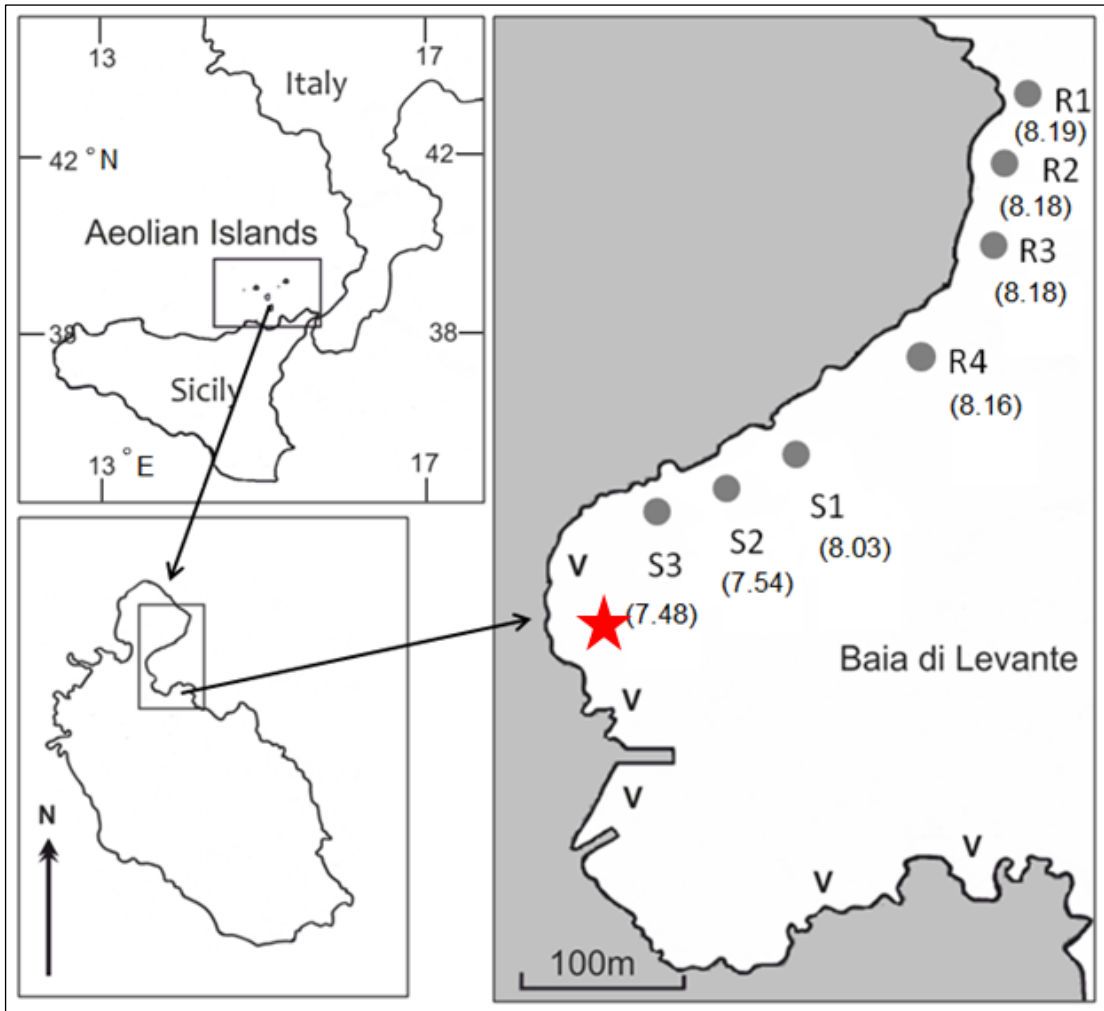
The vents of the Baia di Levante have long been recognised as an important field site for the research of microbial prokaryote extremophiles (Stetter 1988; Amend et al. 2003; Rogers & Amend 2006). However, CO<sub>2</sub> gradients that occur away from the sulphurous vent sources have only recently begun to be exploited to explore the resilience of coastal marine communities to increased *p*CO<sub>2</sub> levels (Arnold et al. 2012; Johnson et al. 2012a, 2012b; Lidbury et al. 2012; Suggett et al. 2012). This chapter presents the results of a study into the carbonate chemistry profile of the CO<sub>2</sub> gradient in the Baia di Levante. It presents a description of the study sites that constitute the basis of the research in subsequent chapters of the thesis and discusses the application of this vent system as a tool in ocean acidification research.

## **2.2 Methodology**

### *2.2.1 Sampling stations*

All physical and chemical sampling occurred along a CO<sub>2</sub> gradient in the northern part of Baia di Levante between September 2009 and May 2012. Within the vent area, three main stations (S1, S2 and S3) were selected for the majority of sampling. These have widely varying pH due to their proximity to the CO<sub>2</sub> vents and were characterised by intermediate to low mean pH (Fig 2.1 & 2.4). The benthos at these sites is visually characterised by fleshy macroalgae, in particular phaeophytes and sea anemones (Appendix I c & d). Three ambient seawater reference stations (R1, R2 and R3) were also selected. These are located outside the vent area and all three have normal, relatively stable mean pH (~ 8.18) with an abundance of calcifying biota (Appendix I e

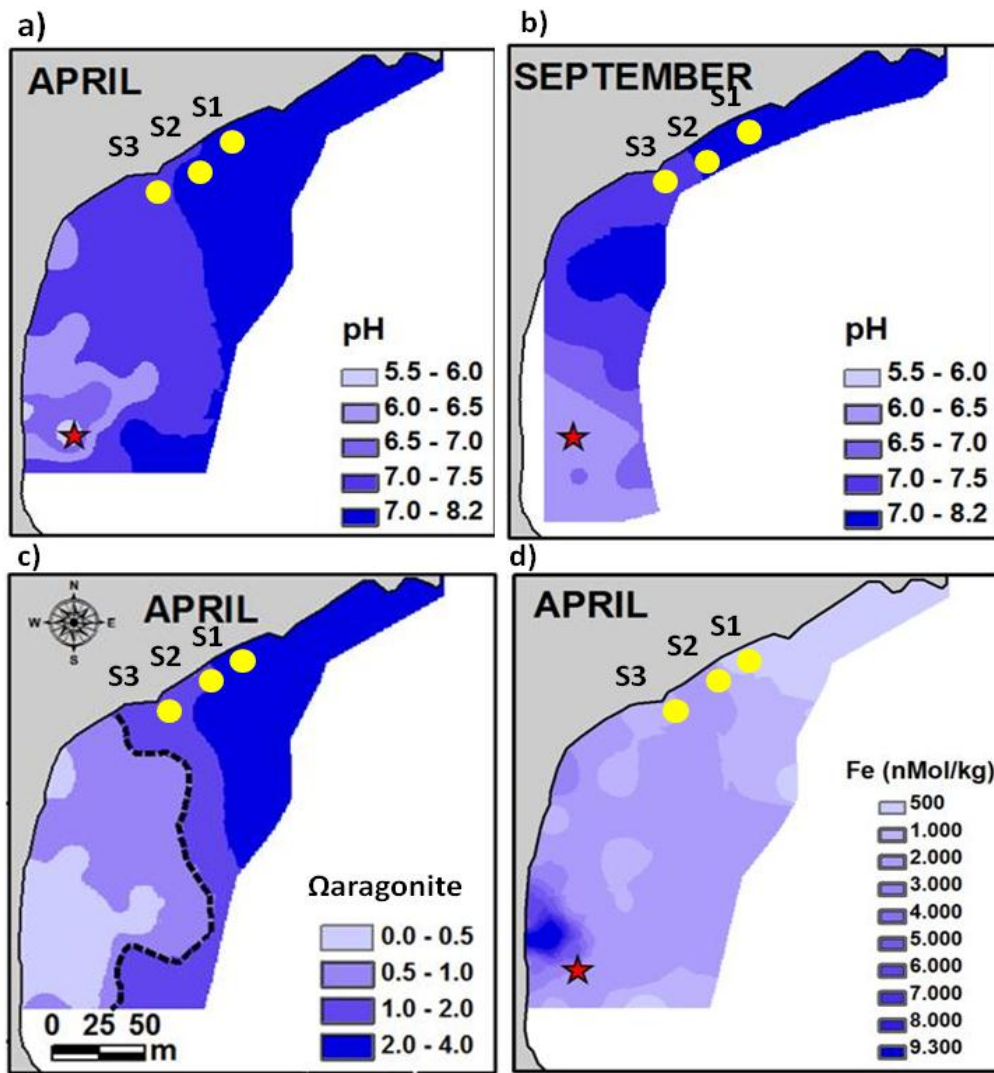
& f). For logistical reasons explained in subsequent chapters, an additional reference station, R4, was used for the studies in Chapter 4 & 5. Station R4 shared a similar pH profile to the other reference stations.



**Fig. 2.1** Map of the study area, Baia di Levante (Vulcano Island, NE Sicily), showing sampling stations S1, S2 and S3, red star = main CO<sub>2</sub> gas vent v = other CO<sub>2</sub> vent sources. Data represent the mean pH of each station ( $n = 23-30$  per station).



**Fig. 2.2** Photographs of Vulcano Island and the study site; the Baia di Levante. Top image shows the active volcano, south facing view. Bottom left image shows CO<sub>2</sub> bubbling at the main gas vent indicated by the red star in the other images. Bottom right image shows the north facing view of Baia di Levante and location of sampling stations.



**Fig. 2.3** Distribution maps adapted from a recent geochemical survey (Boatta et al. in review; permission to reproduce this has been granted by the corresponding author, F.Parello) in the Baia di Levante showing the distinct carbonate chemistry gradient. The locations of the CO<sub>2</sub> enriched sampling stations investigated in this thesis (S1-S3) have been superimposed (yellow dots), the red star indicates the main CO<sub>2</sub> gas vent as also displayed in Figure 1 & 2. Data for these maps were collected from around 70 sampling points within the bay.

- a) pH values measured in the Levante Bay area in April 2011
- b) pH values measured in the Levante Bay area in September 2011
- c) Aragonite saturation states ( $\Omega$ )<sub>a</sub> calculated from the physio-chemical parameters measured in April 2011, the dashes line represents the limit between oversaturated and undersaturated waters
- d) Iron concentrations measured in Levante Bay area in April 2011.

### *2.2.2 Physical and carbonate chemistry measurements*

At all stations a YSI (556 MPS) pH meter was used for rapid measurements of temperature, pH and salinity at a depth of ~ 0.5-1 m. The pH probe was temperature compensated and calibrated (3 point) using National Bureau of Standards (NBS) calibration buffers, probes were recalibrated on a daily basis. A dataset of these parameters was collected for each station from several visits to the study site between September 2009 to May 2012 ( $n = 23-30$ ). Rapid pH fluctuations were recorded along this coastal gradient (over 1 pH unit in under ~ 4 hours observed at S2), so the uncertainty inherent in using the NBS scale for seawater measurements (approximately 0.05 pH, Dickson et al. 2010) was considered acceptable for this study. More accurate measures of pH (i.e. electrometric determination with standard Tris buffer or spectrophotometric determination using indicator dyes) would be required in oceanic systems with more constant, less variable pH values (Dickson et al. 2010).

On the recommendations of Hoppe et al. (2010) total alkalinity (TA) alongside pH was measured to calculate carbonate chemistry. Water samples for TA analysis were collected on three separate occasions (02/10/10, 12/05/11 and 25/09/11). Total alkalinity was measured at each station from a water sample that had been passed through a 0.2  $\mu\text{m}$  pore size filter (stored in the dark at 4 °C). Total alkalinity samples were analysed on an AS-Alk 2 Total Alkalinity Titrator (Apollo SciTech Inc, Georgia, USA) at Plymouth Marine Laboratory (PML). The remaining parameters of the carbonate system;  $p\text{CO}_2$ , DIC,  $\text{HCO}_3^-$  were then calculated with the CO2 SYS software (Lewis & Wallace 1998), using the dissociation constants for carbonic acid supplied by Roy et al. (1993) and the  $\text{KSO}_4$  dissociation constant of Dickson (1990).



This software was also used to calculate the saturation state ( $\Omega$ ) of  $\text{CaCO}_3$ :

$$\Omega = \frac{[\text{Ca}^{2+}] [\text{CO}_3^{2-}]}{K_{sp}}$$

The ( $\Omega$ ) of calcium carbonate is calculated as the product of the concentrations of the reacting ions of  $\text{CaCO}_3$  ( $\text{Ca}^{2+}$  &  $\text{CO}_3^{2-}$ ), divided by the product of the concentration of those ions when the mineral is at equilibrium ( $K_{sp}$ ). If  $\Omega > 1$  seawater is super-saturated and  $\text{CaCO}_3$  precipitation can occur, at  $\Omega < 1$  seawater is under saturated and dissolution can occur.

Light intensity was measured at 1-2 m depth on 27th May 2011 at R2 and S2 using Hobo light loggers (Onset). Water samples for dissolved nutrient analysis (nitrite, nitrate, silicate and phosphate) were collected from stations S1-S3 and R1, < 1 m depth. Samples were collected in 60 ml Nalgene bottles which had been pre-rinsed three times with the sample whilst wearing nitrile-free gloves. Six replicate samples were taken between 25-26<sup>th</sup> May 2011 and frozen prior to colorimetric analysis on a multi-channel Bran Luebbe AutoAnalyser at PML.

### 2.2.3 Statistical analysis

Procedures for the analysis of pH data followed Kerrison et al. (2011). To prevent overestimation of the mean and underestimation of variability, pH data were first transformed to hydrogen ion concentrations. Following statistical testing and calculation of means and interquartile (IQ) ranges, data were re-converted to pH. As this procedure produces a skewed and non-representative deviation around the mean, IQ ranges are more suitable for highly variable data reporting than more common statistical parameters (Kerrison et al. 2011). Differences in pH, TA, light intensities and nutrient concentrations between stations were tested using one-way ANOVA, and multiple

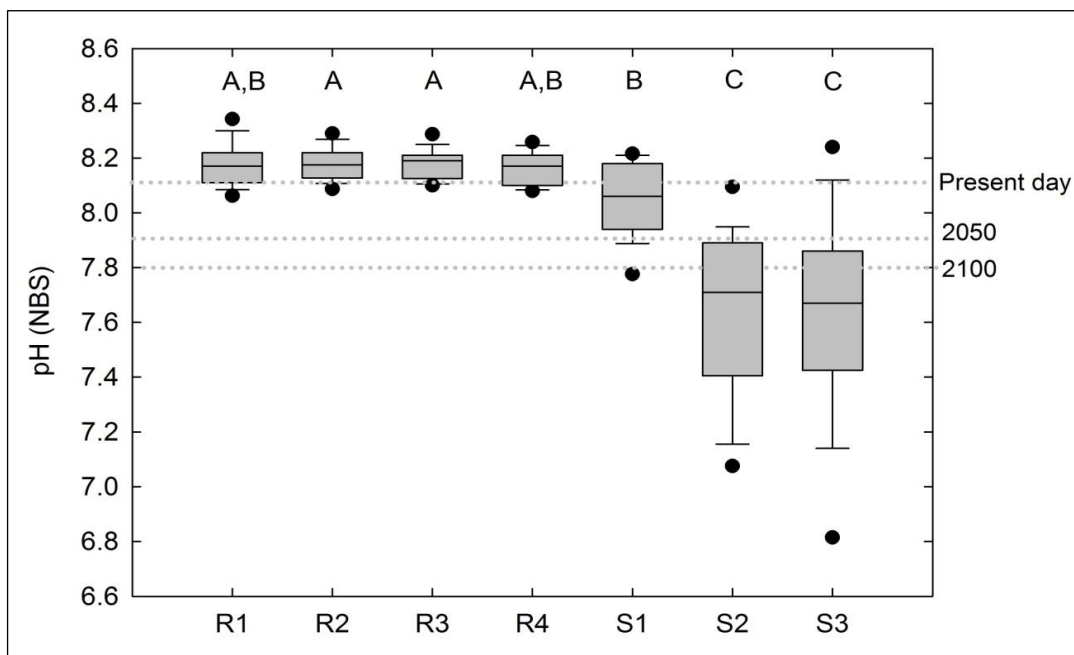
pairwise comparison post hoc procedures (Dunn's Test) were performed when differences were significant. When data failed tests for normality (Shapiro-Wilk) and homogeneity (Levene Median test), and transformations were unsuccessful, Kruskal-Wallis one way analysis of variance on ranks was used. These statistical analyses were performed using SigmaPlot 11.0.

## 2.3 Results

Surface seawater pH significantly decreased with increasing proximity to CO<sub>2</sub> vents at Vulcano (Kruskal-Wallis test,  $H_6 = 108.385$ ,  $P < 0.001$ ; S1 mean = 8.03 IQ: 7.95-8.17; S2 mean = 7.54 IQ: 7.42-7.88; S3 mean = 7.48 IQ: 7.43-7.85,  $n = 27-30$ ). Seawater pH was significantly lower at S2 and S3 than at the reference stations (Dunn,  $P < 0.05$ ) and no significant differences could be detected between R1, R2, R3 and R4 (Dunn,  $P > 0.05$ ). Station S1 had significantly lower pH than R2 and R3 (Dunn,  $P < 0.05$ ) but no significant differences were detected between S1, R1 and R4 (Dunn,  $P > 0.05$ ). The mean pH at the three reference stations fell within the normal range of coastal waters (R1 = 8.19 IQ: 8.11-8.22; R2 = 8.18; IQ: 8.13-8.22; R3 = 8.18 IQ: 8.13-8.21; R4 = 8.16 IQ: 8.10-8.21,  $n = 23-26$ ). Stations closer to the CO<sub>2</sub> vents experienced a greater range in pH than the reference stations, and pH ranges increased with proximity to the vents (Fig. 2.4; S1 range = 0.46, S2 range = 1.03, S3 range = 1.44). A significant difference in pH could not be detected between stations S2 and S3 (Dunn,  $P > 0.05$ ), however, S3 experienced greater fluctuations in pH with recorded minimum readings of 6.80, while the pH at S2 was not recorded below 7.00 (Fig. 2.4).

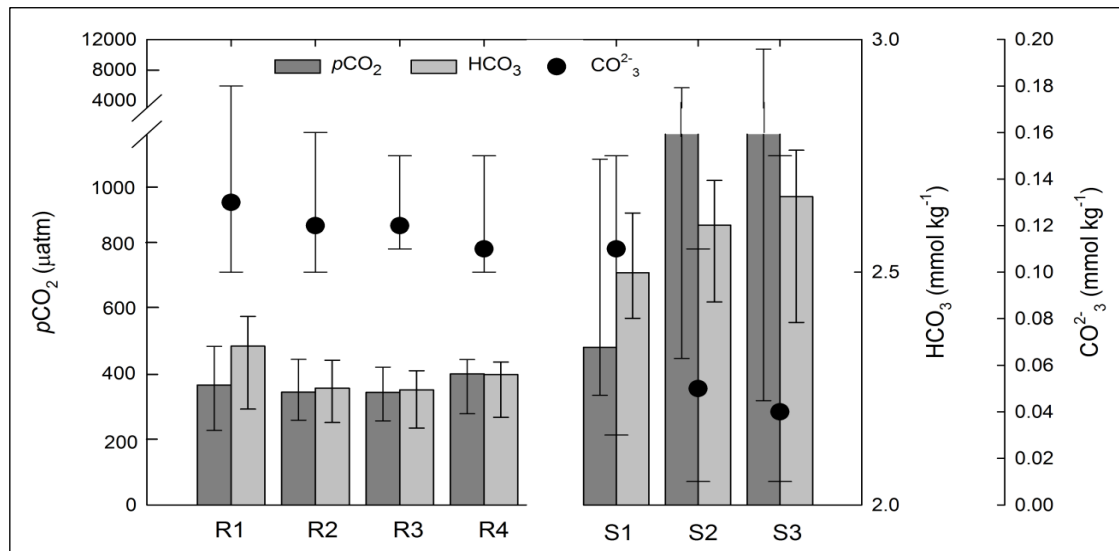
Total alkalinity (2.5-2.7 mmol kg<sup>-1</sup>) did not vary significantly along the CO<sub>2</sub> gradient (ANOVA,  $F_{(6,14)} = 1.46$ ,  $P = 0.261$ ). Salinity (~38) remained relatively constant

between stations, and temperature data matched ambient seasonal fluctuations (18-27 °C). Table 2.1 shows the carbonate chemistry profile of each sampling station. With increasing proximity to the vents,  $p\text{CO}_2$ , DIC and  $(\text{HCO}_3^-)$  increased whilst  $\text{CO}_3^{2-}$  and the saturation of calcite and aragonite decreased (Table 2.1, Fig. 2.5). The highest median values for  $p\text{CO}_2$  and DIC were found at S3 (1428  $\mu\text{tm}$  and 3.7 mmol kg<sup>-1</sup>, respectively), which had the lowest calcite and aragonite saturation levels (1.05 and 0.66  $\Omega$ , respectively). Periods of calcium carbonate under-saturation occurred during the lowest range of pH at stations S2 and S3.



**Fig. 2.4** pH range (NBS scale) of the sampling stations (<0.5 m water depth) along a  $\text{CO}_2$  gradient off Vulcano Island sampled between September 2009 and May 2012 ( $n = 23-30$ ); median = horizontal line, 25th and 75th percentiles = vertical boxes and 10th and 90th percentiles = whiskers, dots = min/max values. Dotted lines represent surface ocean pH under present  $\text{CO}_2$  concentrations and those forecasted for the mid point and the end of this century (IPCC 2007). Post hoc results are indicated by letters; boxes with the same letters are not significantly different ( $P > 0.05$ ).

There was no significant difference found in midday (noon–13:00) light intensities between R2 (mean lux =  $36,935 \pm 3,641$ ,  $n = 13$ ) and S2 (mean lux =  $38,895 \pm 4,234$ ,  $n = 13$ ) (ANOVA:  $F_{(1,24)} = 0.123$ ,  $P = 0.729$ ). Table 2.2 shows the mean nutrient concentrations at stations R1 and S1-S3. Phosphate concentrations remained at low, undetectable levels along the  $\text{CO}_2$  gradient ( $<10$  nmol/L). No significant differences in nitrate concentrations could be detected between stations along the gradient (ANOVA:  $F_{(3,20)} = 1.827$ ,  $P = 0.175$ ). There were significant differences in nitrite (Kruskal-Wallis:  $H_{(3)} = 8.327$ ,  $P < 0.05$ ) and silicate (Kruskal-Wallis:  $H_{(3)} = 12.780$ ,  $P < 0.05$ ) concentrations found between stations. However, post hoc pairwise comparisons revealed that only station S3 had significantly higher nitrite and silicate concentrations than R1 (Dunn,  $P < 0.05$ ); no significant differences in nitrite and silicate could be detected between the remaining stations (Dunn,  $P > 0.05$ ).



**Fig. 2.5** Range in  $p\text{CO}_2$ ,  $\text{HCO}_3^-$  (bar chart) and  $\text{CO}_3^{2-}$  (scatter plot) along a  $\text{CO}_2$  gradient off Vulcano Island, data represent the minimum, median and maximum values determined from the CO2SYS analysis of the long term pH dataset collected between September 2009-May 2012 ( $n = 23-30$ ). Dots and bars = median values, upper & lower limits = maximum and minimum values, respectively.

**Table 2.1** Seawater carbonate chemistry measurements along a CO<sub>2</sub> gradient off the island of Vulcano. Temperature (range 18.6-27.7 °C), pH (NBS scale) and salinity (=38) were measured on several occasions between September 2009 and May 2012. Mean TA ( $\pm$  SE,  $n = 3$ ), pH (min, med & max), temperate, salinity and concentrations of silicate (3.43-19.39  $\mu$ M ) and phosphate (< 10 nmol/L) were used to calculate the remaining parameters using CO2 SYS programme.

Station		pH range: max, med, min (NBS scale)	$p\text{CO}_2$ $\mu\text{atm}$	TA mmol $\text{kg}^{-1}$	DIC mmol $\text{kg}^{-1}$	$\text{CO}_3^{2-}$ mmol $\text{kg}^{-1}$	$\text{HCO}_3^-$ mmol $\text{kg}^{-1}$	$\Omega$ calcite	$\Omega$ aragonite
R1	max	8.35	241	2.682	2.402	0.18	2.206	4.27	2.69
	med	8.17	388	( $\pm 0.12$ )	2.492	0.13	2.341	2.99	1.89
	min	8.06	513		2.538	0.10	2.405	2.39	1.51
R2	max	8.29	274	2.591	2.349	0.16	2.177	3.67	2.31
	med	8.18	365	( $\pm 0.03$ )	2.399	0.12	2.251	2.95	1.86
	min	8.08	471		2.442	0.10	2.311	2.40	1.51
R3	max	8.29	272	2.579	2.337	0.15	2.165	3.65	2.30
	med	8.18	364	( $\pm 0.04$ )	2.394	0.12	2.247	2.94	1.85
	min	8.10	446		2.421	0.11	2.288	2.49	1.57
R4	max	8.26	295	2.582	2.353	0.15	2.189	3.44	2.17
	med	8.12	424	( $\pm 0.05$ )	2.417	0.11	2.280	2.60	1.64
	min	8.08	470		2.438	0.10	2.307	2.40	1.51
S1	max	8.22	355	2.790	2.569	0.15	2.401	3.44	2.17
	med	8.08	510	( $\pm 0.08$ )	2.641	0.11	2.499	2.60	1.64
	min	7.76	1119		2.752	0.06	2.627	1.31	0.82
S2	max	8.10	474	2.742	2.578	0.11	2.436	2.65	1.67
	med	7.71	1244	( $\pm 0.07$ )	2.727	0.05	2.601	1.15	0.73
	min	7.07	5628		3.054	0.01	2.697	0.27	0.17
S3	max	8.24	337	2.796	2.565	0.15	2.392	3.59	2.27
	med	7.66	1428	( $\pm 0.12$ )	3.794	0.04	2.662	1.05	0.66
	min	6.80	10,730		3.428	0.01	2.762	0.15	0.09

**Table 2.2** Mean ( $\pm$  SE) dissolved nutrient concentrations along a CO<sub>2</sub> gradient off the Island of Vulcano ( $n = 5-6$ ). Phosphate was also determined but at all stations was below the detection limit of the AutoAnalyser ( $\sim 10$  nmol/L). Means with the same letters are not significantly different ( $P > 0.05$ ).

Station	Nitrite ( $\mu\text{M}$ )	Nitrate ( $\mu\text{M}$ )	Silicate ( $\mu\text{M}$ )
R1	$0.01 \pm 0.001_{(A)}$	$0.24 \pm 0.05$	$3.43 \pm 0.05_{(A)}$
S1	$0.01 \pm 0.005_{(A,B)}$	$0.13 \pm 0.03$	$8.34 \pm 2.73_{(A,B)}$
S2	$0.02 \pm 0.003_{(A,B)}$	$0.16 \pm 0.06$	$15.12 \pm 2.45_{(A,B)}$
S3	$0.02 \pm 0.001_{(B)}$	$0.33 \pm 0.1$	$19.39 \pm 1.24_{(B)}$

## 2.4 Discussion

The subterranean CO<sub>2</sub> vent activity in the shallow water of the Baia di Levante produces a pH gradient from mean pH 8.1-8.2 to  $\sim 7.4$ , over a  $\sim 300$  m stretch of rocky coastline. Along this gradient seawater gradually becomes sub-saturated with calcite and aragonite as  $p\text{CO}_2$  concentrations elevate approaching the vents. This is consistent with the findings from a recent geochemical survey of the bay (Boatta et al. in review; Fig 2.3). Due to dynamic vent activity and advection of high CO<sub>2</sub> water, the stations close to the CO<sub>2</sub> vents were characterised by high pH variability as also reported in other vent studies (Fabricius et al. 2011; Kerrison et al. 2011; Boatta et al. in review). Several sampling stations with contrasting carbonate chemistry profiles can be studied along this gradient, to test ecosystem-scale predictions of ocean acidification scenarios. This is comparable to the approach recently adopted at vent sites in the Bay of Naples (Hall-Spencer et al. 2008; Porzio et al. 2011) and in Papua New Guinea (Fabricius et al. 2011;

Uthike & Fabricius 2012). Extending the use of naturally CO<sub>2</sub> enriched sites over a variety of different regions and habitats allows for comparison of biological and ecological trends between different ecosystems with similar carbonate chemistry gradients thereby strengthening wider scale predictions of the impacts of ocean acidification on marine ecosystems (Johnson et al. 2012b).

Potential confounding variables such as light, temperature, tidal amplitude/wave exposure, depth, total alkalinity and salinity were homogenous between stations increasing the likelihood that biological and ecological responses may be attributed to pH/CO<sub>2</sub> levels. There are, however, some variations in benthic habitat characteristics along the gradient; there is an increase in the amount sandy sediment approaching the vent sources, whilst the reference stations are predominantly characterised by rocky benthos. This therefore needs to be considered when designing experiments along the gradient and for the interpretation of results. The fact that the alkalinity of CO<sub>2</sub> enriched stations was similar to ambient values favours the use of these types of naturally acidified sites over the use of others, such as the low pH springs in Mexico (Crook et al. 2012), which experience high and variable alkalinity that is unrepresentative for future oceans subjected to acidification. Dissolved nutrient levels are, however, a potentially important confounding variable to consider in these *in situ* experiments as it is difficult to isolate the effects of nutrient enrichment from the effects of CO<sub>2</sub> elevations on algal growth. Along the Vulcano CO<sub>2</sub> gradient phosphate and nitrate levels were similar between all stations, and the increase in nitrite at stations S2 and S3 was only very small (0.01 µM). However, there was an increasing trend in silicate concentrations with increasing proximity to the CO<sub>2</sub> vents despite statistically significant differences only being detected between R1 and S3. The larger amounts of sandy sediment habitats in stations S2 and S3 relative to the reference stations may explain the increase in silicate as marine sediments can release dissolved silica into silica-poor seawater (Wiley 1978).

In addition, submarine vent fluids may be enriched in dissolved silicate (Dando et al. 1999). The potential effects of the increasing trend in silicate concentration along the CO<sub>2</sub> gradient on diatom responses are discussed in Chapter 3. Thus, although some nutrients were slightly elevated at S3, the major physical gradient across the stations appeared to be the increase in concentration of *p*CO<sub>2</sub> and lowering of carbonate saturation states as pH levels significantly decreased. The changes observed in algal communities between stations (Chapters 3-6) are likely to be affected by the large variations in carbonate chemistry caused by the CO<sub>2</sub> vent activity.

Marine volcanic vents often have high levels of toxic compounds and can sometimes have hypoxic waters (Kelley et al. 2002; Tunnicliffe et al. 2009) which produces confounding effects so that the biological patterns cannot be attributable to the effects of CO<sub>2</sub> alone. Along the Vulcano CO<sub>2</sub> gradient however, hydrogen sulphide and oxygen concentrations did not vary significantly (Boatta et al. in review). The concentration of iron, a potential limiting nutrient for algae, is elevated in the area of stations S1-S2 relative to R1-R3 (Boatta et al. in review; Fig 2.3d). Care is therefore needed, to discriminate the effects of CO<sub>2</sub> and iron on algal growth. However, elevations in iron within the CO<sub>2</sub> enriched area studied (S1-S3) did not increase in the same linear fashion as *p*CO<sub>2</sub>. Furthermore, iron concentrations remain constant between the reference station and the CO<sub>2</sub> enriched station S1, so this does not confound biological responses attributed to CO<sub>2</sub> between these stations. Acidification of coastal seawater can enhance iron bioavailability through pH induced changes in iron chemistry (Breitbarth et al. 2010) and increase concentrations of one of the most toxic and bioavailable forms of copper (Richards et al. 2011). Therefore observations of algal assemblages made along the Vulcano CO<sub>2</sub> vent gradient may provide insights into future trends, as elevated seawater concentrations of iron (and other metals) are expected in a future high CO<sub>2</sub> world.



In this thesis, differences in pH and seawater carbonate chemistry between sampling stations have been compared with changes in marine biota, yielding correlations between CO<sub>2</sub> enrichment and coastal ecosystem responses. Carbon dioxide vents acidify seawater to levels that are expected to be seen by the end of this century and beyond (Caldeira & Wickett 2005). Median CO<sub>2</sub> levels at S1 are comparable to conditions forecasted for near-future acidification around the middle of this century (Nakićenović & Swart 2000; Feely et al. 2009), whilst median values and range at S2 and S3 go beyond those predicted for the end of this century. These extreme low pH stations, however, remain useful for examining organism response and tolerance boundaries over a wide range of pH values (Barry et al. 2010). Furthermore, they can be used for predicting the impacts of a range of potential ocean acidification scenarios in which localised areas of seawater will be exposed to levels of CO<sub>2</sub> extending beyond the worst case scenarios predicted for surface oceans. These include those areas that have the potential to be exposed to dramatic CO<sub>2</sub> leaks following deep sea sequestration (Caldeira et al. 2005; Blackford et al. 2009), enhancement of acidification by eutrophication events (Cai et al. 2011), upwelling areas of CO<sub>2</sub> rich seawater (Diaz & Rosenberg 2008; Feely et al. 2008; Thomsen et al. 2010) and hypoxic conditions (Bograd et al. 2008; Brewer & Peltzer 2009). This is particularly true in coastal zones, where due to the spread of hypoxic zones, the magnitude of expected changes in  $p\text{CO}_2$  may be higher than previously thought (Melzner et al. 2012).

There are drawbacks with using CO<sub>2</sub> vents systems as proxies for future ecological change due to ocean acidification. Being open systems, their ecology is affected by surrounding areas that have lower CO<sub>2</sub> levels, allowing recruitment and migration of organisms from unaffected habitats (Fabricius et al. 2011; Gazeau et al. 2011). Volcanic vent sites can have highly variable CO<sub>2</sub> levels and are characterised by steep gradients in pH and carbonate saturation. High variability in the carbonate system may be

considered a drawback to *in situ* studies because accurate dose-response relationships become difficult to determine. Furthermore, surface waters are not thought to be characterised by such rapid variability as the oceans acidify (Riebesell et al. 2008) and this may complicate the use of the information derived from vent studies in projecting future high CO<sub>2</sub> scenarios (Gazeau et al. 2011). In addition, care is required when designing experiments along these gradients to avoid unrealistic confounding influences of other factors such as potential elevations in metals and toxic compounds, variations in nutrients and decreases in oxygen content. Despite these drawbacks, CO<sub>2</sub> vents can augment predictions based on laboratory and modelling experiments since they show which organisms can tolerate exposure to increases in CO<sub>2</sub> levels at a variety of locations worldwide (Wernberg et al. 2012).

CO<sub>2</sub> vent systems also provide an opportunity to examine the ecological effects of pH variability. This is essential for forecasting organism responses to acidification in habitats exposed to large natural diel, semi-diurnal and stochastic fluctuations in the carbonate system. The pH of the oceans, particularly coastal regions, is not constant (Middelboe & Hansen 2007; Joint et al. 2011), over diurnal scales pH shows strong systematic variation as a result of CO<sub>2</sub> uptake during photosynthesis and CO<sub>2</sub> release during respiration (Wootton et al. 2008). A recent compilation of high resolution time series of upper ocean pH collected over a variety of ecosystems has highlighted the natural temporal fluctuations (over a period of one month) and environmental heterogeneity associated with coastal seawater pH (Hoffmann et al. 2011). This natural variability was seldom considered in the early stages of ocean acidification research, as perturbation experiments mainly investigated the responses of organisms to constant levels of lowered pH. It is important to incorporate natural pH variability in ocean acidification studies, as ambient fluctuations in pH may have a large impact on the development of resilience in marine populations, regulating organism performance

(Hoffman et al. 2011). Volcanic vent systems are useful as they can reveal ecological responses to long-term moderate increases in CO<sub>2</sub> levels that retain natural pH variability (Fabricius et al. 2011; Kerrison et al. 2011). The installation of the recently developed semi-permanent pH sensors (Seidel et al. 2008; Martz et al. 2010; Boatta et al. in review) will improve the accuracy in determining the levels of temporal variability within CO<sub>2</sub> vent systems.

Despite some recent attempts to investigate the ecological impacts of elevated CO<sub>2</sub> on species interactions (Bibby et al. 2007; Diaz-Pulido et al. 2011; Doropoulos et al. 2012; Johnson & Carpenter 2012; Kroeker et al. 2012; Landes & Zimmer 2012), progress in understanding potential impacts of ocean acidification on marine systems is still limited by the scarcity of information at the community and ecosystem level (Gazeau et al. 2011). Naturally CO<sub>2</sub> enriched sites can serve as valuable proxies for future changes in benthic environments, as they incorporate natural ecosystem dynamics and ecological interactions. Despite certain limitations, this mensurative approach provides insights that are complimentary in scope and scale to the prevalent *ex situ* approaches (Wernberg et al. 2012). The CO<sub>2</sub> vent gradient described in this chapter forms the basis of the research approach adopted in the following chapters. Across the defined sampling stations along the CO<sub>2</sub> gradient, ecological and physiological changes in various benthic marine algae are investigated in order to explore the *in situ* effects of elevated CO<sub>2</sub> levels on the productivity, structure and function of benthic ecosystems.

### **Chapter 3: Colonisation of Periphyton Assemblages on Artificial Substrata Along a Natural CO<sub>2</sub> Gradient**

The colonisation of artificial substrata by microalgal assemblages (periphyton) was examined *in situ* along a natural marine CO<sub>2</sub> gradient. Periphyton communities altered significantly as CO<sub>2</sub> concentrations increased. CO<sub>2</sub> enrichment correlated with increases in Chl *a* concentrations and with increases in diatom abundance, although no changes were detected in cyanobacteria at the levels of CO<sub>2</sub> predicted for this century. Scanning electron microscope analysis revealed major shifts in diatom assemblage composition as CO<sub>2</sub> levels increased. These results show that changes in phototrophic biofilms due to rising anthropogenic CO<sub>2</sub> emissions may have significant ecological ramifications for coastal systems with potential economic repercussions for marine industries.

The data from this chapter have been published online and are due to occur in print in a special edition of the journal *Marine Biology* in 2012:

**Johnson VR**, Brownlee, C, Rickaby REM, Graziano M, Milazzo M & Hall-Spencer, JM (2012a) Responses of marine benthic microalgae to elevated CO<sub>2</sub>. *Marine Biology*, DOI: 10.1007/s00227-011-1840-2

A follow-up paper examining microbial colonisation of glass slides has also been published:

Lidbury I, **Johnson VR**, Hall-Spencer JM, Munn CB, Cunliffe M (2012) Community-level response of coastal microbial biofilms to ocean acidification in a natural carbon dioxide vent system. *Marine Pollution Bulletin* 64: 1063-1066

### 3.1 Introduction

Natural and artificial substrata in the marine environment are colonised by microbial biofilms. These can be defined as surface-associated layers of microbial cells embedded in an extracellular polymeric substance (EPS) (Characklis & Marshall 1989). They are composed of bacteria, protozoans, and in intertidal and shallow subtidal habitats, are characterised by high population densities of periphyton. Periphyton are defined as microflora living attached to the surfaces of submerged objects (Azim et al. 2005) and in marine biofilms consist mainly of diatoms and cyanobacteria. Benthic diatoms, in particular, are major foulers of artificial surfaces in the marine environment (Edyvean & Moss 1986; Molino & Wetherbee 2008).

Photoautotrophic microbial biofilms contribute significantly to primary productivity (Hawkins et al. 1992; Bustamante et al. 1995) and are considered an important functional component of the benthos in marine ecosystems (Underwood 1984a; Hill & Hawkins 1991; Thompson et al. 2004). These communities are a valuable food source for a variety of marine organisms (Underwood 1984b; Abreu et al. 2007; Kuwae et al. 2008), representing an important trophic link between the water column and the higher trophic levels of an ecosystem (Hynes 1970).

Biofilms are ubiquitous communities and their development is considered the first stage of succession in the marine environment (ZoBell & Allen 1935; McCook & Chapman 1993). They play important roles in determining the structure and dynamics of the overlying benthic communities by enhancing and/or inhibiting the settlement of invertebrates and macro algal propagules (Crisp & Ryland 1960; Meadows & Williams 1963; Huang & Boney 1984; Wicczorek et al. 1996; Thompson et al. 1998). 'Bio-fouling' is the unwanted colonisation of biofilms and the subsequent settlement of macroalgal propagules and invertebrates on man-made surfaces in the marine

environment. This can cause serious problems for marine industries and navies around the world (Yebra et al. 2004). Biofilms are known to encourage the corrosion of metals and concrete (Callow & Edyvean 1990) and reduce the speed of ships by up to 15 % (Lewthwaite et al. 1985).

The factors regulating the establishment and development of biofilms have been extensively studied, including substrata type (Becker & Wahl 1991; Totti et al. 2007), season (Castenholz 1961; Hill & Hawkins 1991), grazing (Anderson 1995; Thompson et al. 2000), spatial variation (Underwood 1984a; MacLulich 1987), nutrients (Keithan & Barnese 1989; Decho 2000; Sanz-Lázaro et al. 2011) and pH (Sekar et al. 2004). The potential impact of elevated CO<sub>2</sub> on these communities however, is poorly understood. Most of the research to date has focussed on the responses of oceanic diatom and cyanobacteria species (Riebesell et al. 1993; Burkhardt et al. 2001; Levitan et al. 2007; Sun et al. 2011) and mixed phytoplankton assemblages to elevated CO<sub>2</sub> (Tortell et al. 2002, 2008; Kim et al. 2006; Trimborn et al. 2009). More recently, Torstensson et al. (2011) reported a small (5 %) reduction in growth rates in the polar benthic diatom, *Navicula directa* and Witt et al. (2011) observed CO<sub>2</sub> induced shifts in biofilm microalgal communities over a short term laboratory experiment. The response of benthic microalgae assemblages to CO<sub>2</sub> enrichment *in situ*, however, has yet to be addressed.

This chapter presents the results of an investigation that compared periphyton assemblages on artificial substrata installed along a coastal CO<sub>2</sub> gradient off the island of Vulcano, NE Sicily. The aim of this study was to determine the responses of these microalgal assemblages to elevations in CO<sub>2</sub> and to characterise any changes in the diatom and cyanobacteria populations to better understand and predict the ecological effects of ocean acidification on photoautotrophic biofilms.

## 3.2 Methodology

### 3.2.1 Study sites and carbonate chemistry

This study was conducted between 16<sup>th</sup> September and 8<sup>th</sup> October 2010 along a CO<sub>2</sub> gradient off the island of Vulcano in Sicily. Experiments for this chapter were carried out at stations R2 (mean pH 8.18), S1 (mean pH 8.03) and S2 (mean pH 7.54).

See Chapter 2 for full site and station description and the carbonate chemistry methodology used.

### 3.2.2 In situ sampling

At each station perspex slides (75 mm x 25 mm x 2 mm) were attached horizontally to four anchored floats (6 slides per float placed at 25 mm intervals), suspended in the shallow subtidal zone (< 1 m depth) ( $n = 24$  slides per station). Perspex (hydrophobic surface) was selected over glass (hydrophilic) to maximise the amount of microalgal attachment (Sekar et al. 2004). The slides were removed after 21 days by which time they had established biofilms which were visible to the naked eye. Half of the slides ( $n = 12$  per station) were chosen randomly for chlorophyll extraction. They were immediately preserved at -20 °C (for < 48 hours) and stored at -80 °C upon return to the laboratory (for < 2-3 weeks) to prevent chlorophyll degradation (Thompson et al. 1999). The remaining slides were fixed in glutaraldehyde (2.5 % with filtered seawater) for 1 hour in the dark, rinsed and then frozen as above until epi-fluorescence analysis (< 1 month) and viewing under the scanning electron microscope (SEM).

### 3.2.3 Sample Analysis

#### 3.2.3.1 Chlorophyll *a* extraction

The amount of Chl *a* present within a biofilm is a useful proxy for the photosynthetic standing crop, therefore its extraction and spectrophotometric quantification can provide a reliable but relative indication of total photosynthetic biomass and productivity (Underwood 1984a,c). The photosynthetic standing stock of each slide ( $n = 12$ ) was measured from chlorophyll extracted in 100% boiling ethanol ( $\sim 70^\circ\text{C}$ ) for two minutes. Ethanol was chosen as the solvent for this experiment as it is an efficient extractant of chlorophyll from resistant material and provides the most reliable estimates for use with natural assemblages of mixed microalgae (Ritchie 2008). The absorbance of each sample at 632, 649, 665, 696 and 750 nm, was measured using a Cecil CE2011 spectrophotometer and the concentration of the total Chl *a* in the sample ( $\mu\text{g cm}^{-2}$ ) was calculated using the quadrichroic equation of Ritchie (2008).

#### 3.2.3.2 Epi-fluorescence

The relative abundance of cyanobacteria and diatoms within the periphyton on each slide ( $n = 12$ ) was determined by epi-fluorescence using confocal laser scanning microscopy (CLSM). It is possible to differentiate between biofilm photoautotrophic members based on differences in their chlorophyll autofluorescence. In this way CLSM has been successfully applied to examine and visualise the structure and growth of mixed species photoautotrophic biofilms (Nagarkar & Williams 1997; Norton et al. 1998; Neu et al. 2004). Bright-field light microscopy is not a recommended technique for quantifying cyanobacteria in biofilms as only a few types can be clearly observed (Nagarkar & Williams 1997). Cyanobacteria disperse their pigments throughout their



cytoplasm (as opposed to diatoms where they are enclosed in plastids) thereby making epi-fluorescence a suitable technique for quantifying accurate areal coverage of these microorganisms within a biofilm. In addition, diatom cells were difficult to count directly with bright-field light microscopy (thick films on some slides prevented adequate passage of light) so in this study areal coverage of their epi-fluorescence was used as proxy for abundance. Slides were viewed using a Radiance 2000 CLSM (Bio-Rad, UK), excitation 488 nm; emission 570-590/70 and 660-700 nm. Thirty images at a fixed low power magnification (x 10) were taken at random locations across each slide and the average percentage cover of cyanobacteria and diatom fluorescence was digitally quantified using Image J software (v 1.43, National Institutes of Health, Bethesda, MD, USA).

#### *3.2.3.3 SEM Analysis*

The composition of the attached diatom assemblage from five replicate slides at each station was analysed by SEM. Slides were cut into  $\sim 1 \text{ cm}^2$  pieces, air dried (Hill & Hawkins 1990) and coated with gold prior to observation with a JEOL JSM 5600 LV SEM. Each slide was examined at fixed magnification (x 500) and the abundance of different diatom genera (identified according to Round et al. 1990) was recorded from counts in ten randomly positioned photographs on the colonised areas of the slide. Diatoms that could not be accurately identified were assigned to numbered groups (i.e unidentified pennate 1, 2, unidentified naviculoid 1).

### 3.2.4 Statistical Analysis

Differences in periphyton assemblages among stations were tested using one-way ANOVA and multiple pairwise comparison post hoc tests (Tukey's Test) were performed where differences were significant. Data that failed tests for normality (Shapiro-Wilk) and homogeneity (Levene Median test) were transformed; arc sin (suitable for data on proportions) and exponential (effective at transforming skew unimodal distributions into nearly symmetric normal-like distributions). When transformations were unsuccessful, data was analysed by Kruskal-Wallis one way analysis of variance on ranks and multiple pairwise comparison post hoc tests (Dunn's method). These statistical analyses were performed using SigmaPlot 11.0.

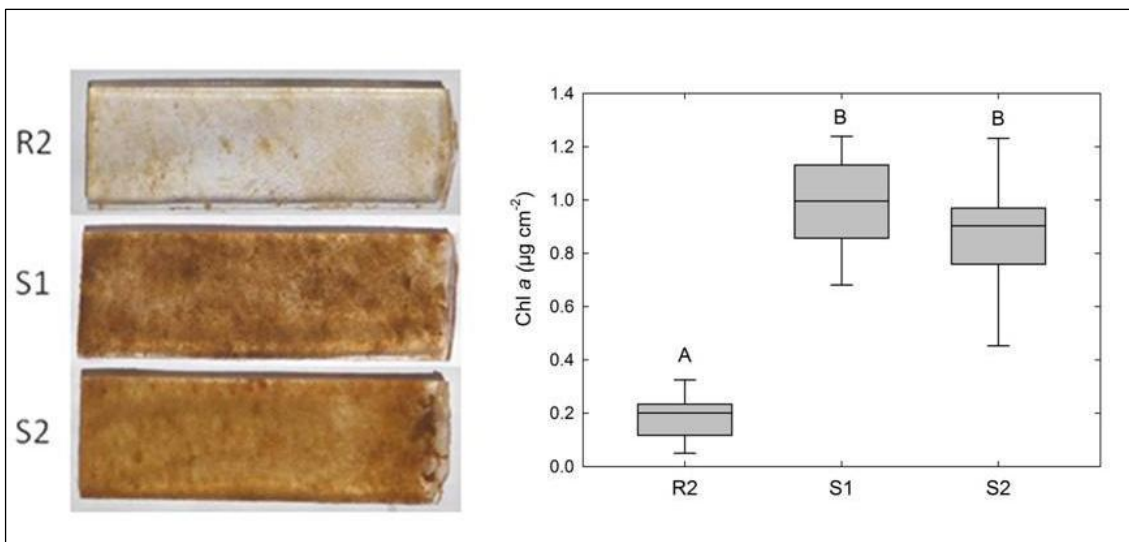
The abundances of diatom genera were used to calculate Shannon diversity (Shannon & Weaver 1949), Pielou's evenness (Pielou 1975) and Simpsons index of dominance (Simpson 1949) for each slide. The similarity of diatom assemblages across the different slides ( $n = 15$ ) was examined by hierarchical cluster analysis using IBM SPSS Statistics 18. Only genera representing over 1% abundance were included in this analysis including any of the numbered unidentified diatoms groups. Assemblages were clustered using a dissimilarity coefficient (squared Euclidian distance) and Ward's method (minimum variance clustering).

## 3.3 Results

There was an increase in Chl *a* concentrations on the slides from the CO<sub>2</sub> enriched stations S1 and S2 relative to the reference station R2. The highest values were measured on slides from S1 ( $0.99 \mu\text{gcm}^{-2} \pm 0.05$ ) which were almost five-fold higher than those at R2 ( $0.19 \mu\text{gcm}^{-2} \pm 0.03$ ). Mean Chl *a* concentrations were significantly

different between stations (Fig 3.1; ANOVA:  $F_{(2,33)} = 69.412$ ,  $P < 0.001$ ). Slides from S1 and S2 contained significantly greater Chl *a* concentrations than R2 (Tukey,  $P > 0.05$ ) but no significant difference could be detected between S1 and S2 (Tukey,  $P < 0.001$ ).

Diatom abundance (mean % cover on slides) was greater in the CO<sub>2</sub> enriched stations S1 and S2 relative to the reference station R2. The highest abundances were found on slides at S2 (60 %  $\pm$  1.11), a seven-fold increase from R2 (8.5 %  $\pm$  0.60). A significant difference in diatom abundance was detected between stations (Fig 3.2; ANOVA:  $F_{(2,33)} = 610.212$ ,  $P < 0.001$ ); with significant increases recorded with increasing CO<sub>2</sub> levels (Tukey,  $S2 > S1 > R2$ ,  $P < 0.001$ ). There was no significant difference in the percentage cover of cyanobacteria in the periphytic assemblages between stations (Fig 3.2; ANOVA:  $F_{(2,33)} = 3.041$ ,  $P = 0.061$ ) which remained relatively low (< 2 %) along the gradient.

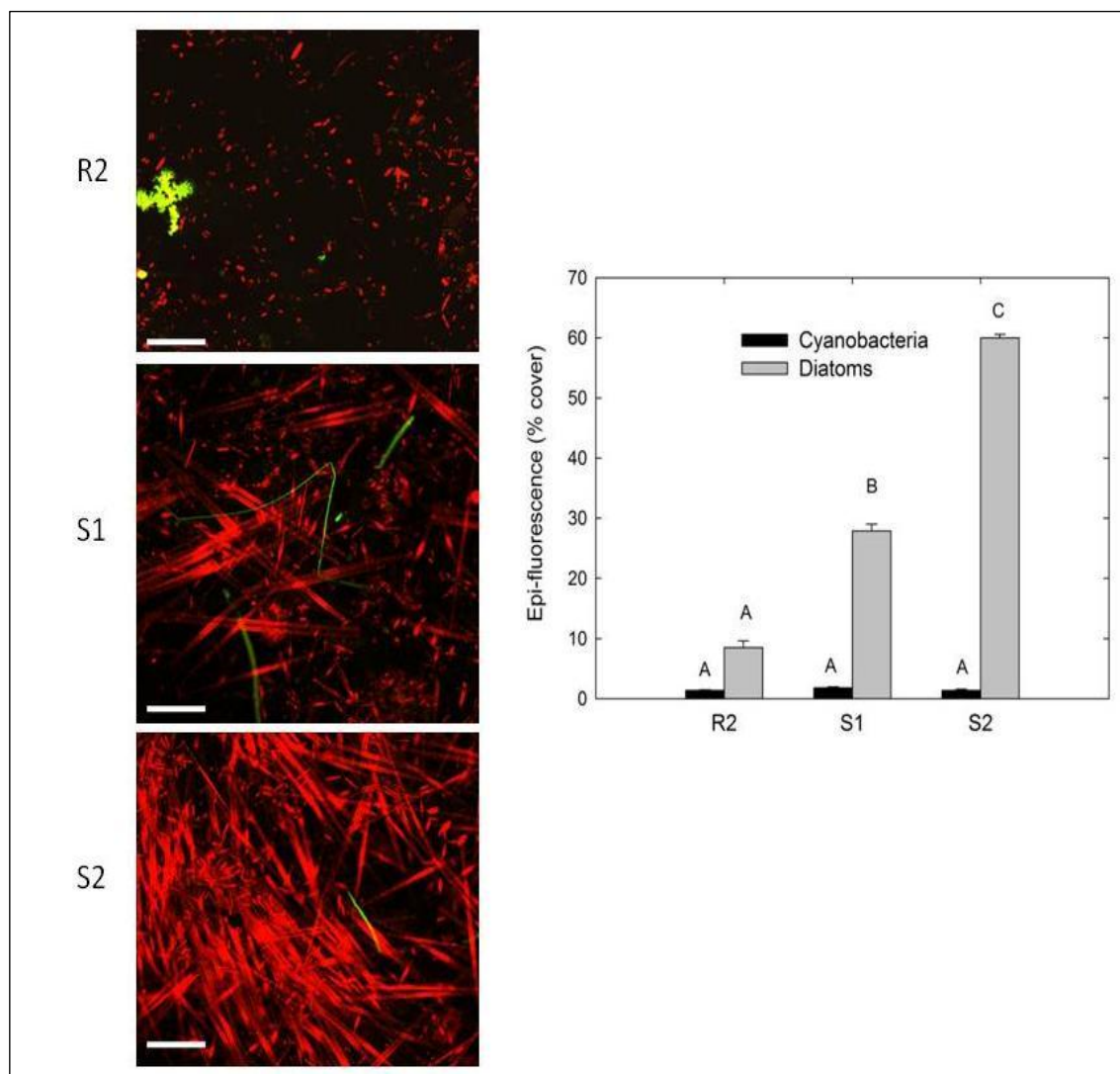


**Fig. 3.1** Images of biofilms that colonised the slides after three weeks at S1 S2 and S3 along the Vulcano CO<sub>2</sub> gradient. Box plot shows Chl *a* concentration ( $\mu\text{g cm}^{-2}$ ) of colonised slides at S1-S3 ( $n = 12$  per station). Median = horizontal line, 25th and 75th percentiles = vertical boxes and 10th and 90th percentiles = whiskers. Boxes with the same letter are not significantly different ( $P > 0.05$ ).

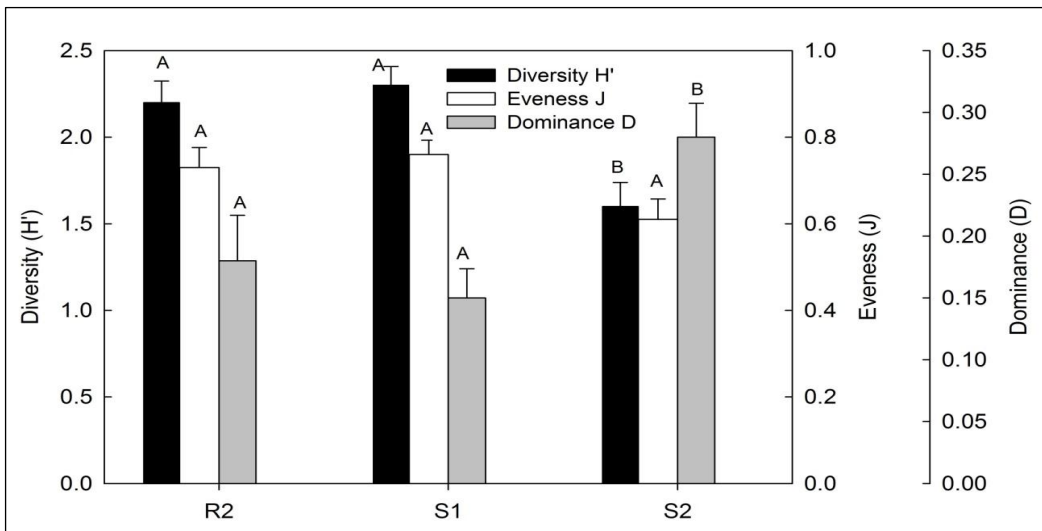
The mean generic diversity ( $H'$ ) of periphytic diatom assemblages decreased under the highest  $p\text{CO}_2$  concentration at S2. Mean diversity was significantly different between stations (Fig 3.3; ANOVA:  $F_{(2, 12)} = 9.347$ ,  $P < 0.001$ ). S2 had a significantly lower diversity ( $1.59 \pm 0.14$ ) than S1 and R2 (Tukey,  $P < 0.05$ ) but no differences in diversity could be detected between S1 and R2 (Tukey,  $P > 0.05$ ,  $2.3 \pm 0.11$ ,  $2.2 \pm 0.28$ , respectively). The dominance index (D) increased at S2. There were significant differences recorded in dominance between stations (ANOVA:  $F_{(2,12)} = 5.147$ ,  $P = < 0.05$ ) with significantly higher values at S2 than S1 (Tukey,  $P < 0.05$ ,  $0.28 \pm 0.03$ ,  $0.15 \pm 0.02$ , respectively), however, no significant difference could be detected between the remaining stations (Tukey,  $P > 0.05$ ). Mean evenness ( $J'$ ) was the lowest in S2 (0.61), however no significant differences could be detected between the stations (ANOVA:  $F_{(2,12)} = 3.678$ ,  $P = 0.057$ ).

Cluster analysis of relative abundances of periphytic diatom genera for each slide yielded four separate groups (Fig. 3.4). The slides from R2 formed widely separated, distinct groups whilst slides from S1 and S2 were more closely linked indicating greater similarities in assemblage compositions. Figures 3.5 & 3.6 highlight the marked differences in assemblage composition between the  $\text{CO}_2$  enriched stations (S1 & S2) and the reference station (R2). A dramatic shift in assemblage composition occurred as the relative abundance of *Toxarium* and *Grammatophora* increased from 2.1 % and 0.5 % respectively in the R2 assemblages to 41 % and 24 % in S2. Figure 3.7 displays mean cell counts of the most numerous taxa from the SEM images but cannot be accurately scaled-up for whole slide totals as there was a higher incidence of uncolonised spaces at R2. It shows that the changes in carbonate chemistry between the stations appear to relate to the increased abundance of some genera (viz.; *Toxarium*, *Grammatophora*, *Bacillaria*, *Navicula*, *Cocconeis*, *Amphora*). There were significant increases

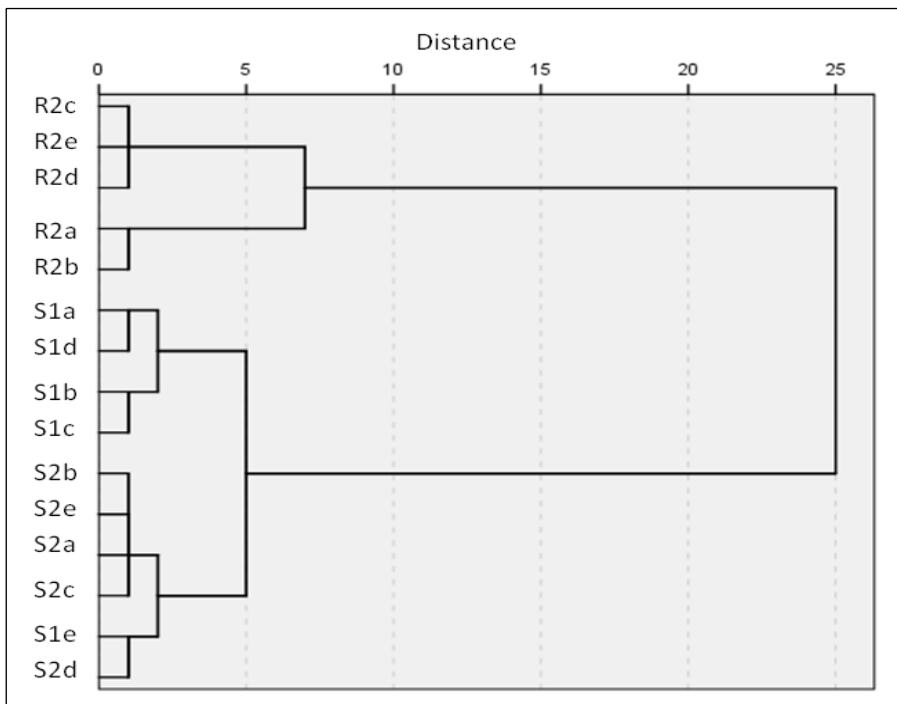
in *Toxarium* (ANOVA:  $F_{(2, 12)} = 25.132$ ,  $P < 0.001$ ), *Grammatophora*, (ANOVA:  $F_{(2, 12)} = 7.910$ ,  $P < 0.001$ ) and *Bacillaria* (ANOVA:  $F_{(2, 12)} = 7.968$ ,  $P < 0.001$ ), , in both the CO<sub>2</sub> enriched stations relative to the reference station. The abundance of other genera decreased with increasing CO<sub>2</sub> / decreasing pH (viz.; *Cyclophora*, *Neosynedra*, *Rhabdonema*, *Nitzschia*). There were significant reductions in *Cyclophora* (ANOVA:  $F_{(2, 12)} = 6.923$ ,  $P < 0.05$ ), *Neosynedra* (ANOVA:  $F_{(2, 12)} = 70.071$ ,  $P < 0.001$ ), *Rhabdonema* (ANOVA:  $F_{(2, 12)} = 4.729$ ,  $P < 0.05$ ) and *Nitzschia* (ANOVA:  $F_{(2, 12)} = 4.847$ ,  $P < 0.05$ ) along the pH/CO<sub>2</sub> gradient. Some genera, on the other hand, did not appear to be affected by the changes in pH/CO<sub>2</sub> such as *Licmophora* (ANOVA:  $F_{(2, 12)} = 1.489$ ,  $P = 0.264$ ).



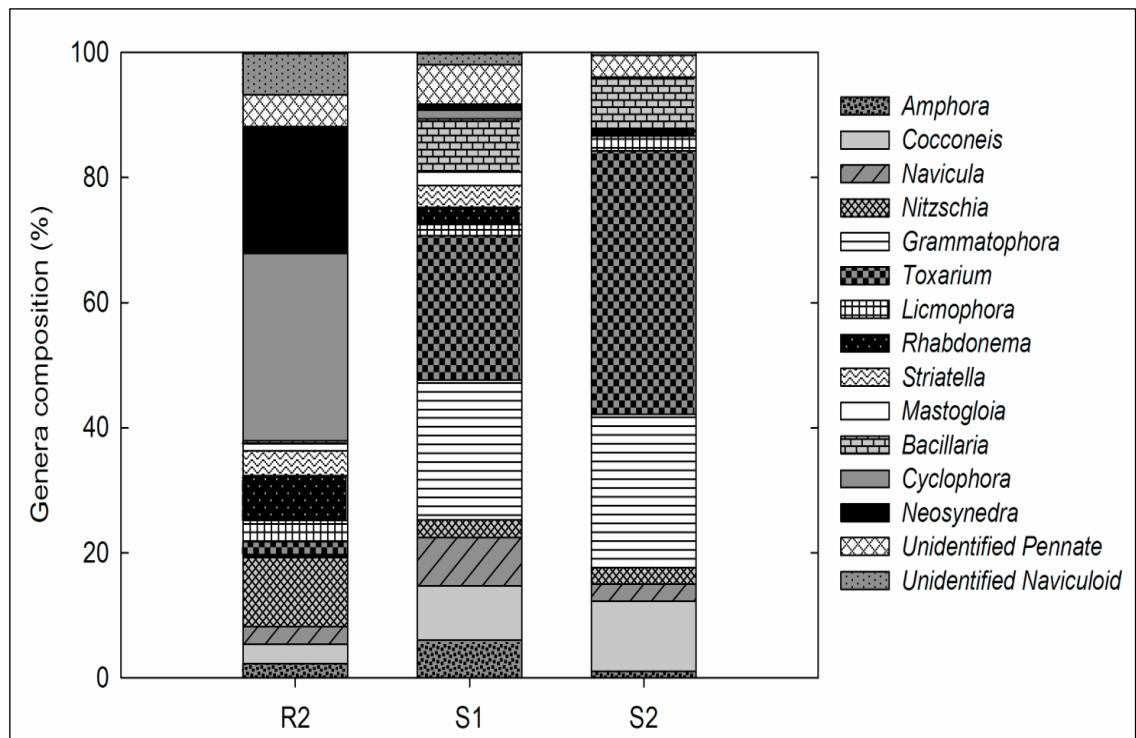
**Fig. 3.2** Epi-fluorescence microscope images of slides colonised in R2, S1 & S3. Red = diatom chlorophyll fluorescence, Green / yellow = cyanobacteria (scale bars = 50 μm). Graph shows relative percentage cover (based on chlorophyll epi-fluorescence) ± SE of cyanobacteria and diatoms along the CO<sub>2</sub> gradient ( $n = 12$  per station). Means with the same letters are not significantly different ( $P > 0.05$ ).



**Fig. 3.3** Mean  $\pm$  SE ( $n = 5$  per station) for diversity (Shannon  $H'$ ), evenness (Pielou's  $J'$ ) and dominance (Simpsons index  $D$ ) of periphytic diatom assemblages along the Vulcano  $CO_2$  gradient. Means with the same letters are not significantly different ( $P > 0.05$ ) within each metric across stations.

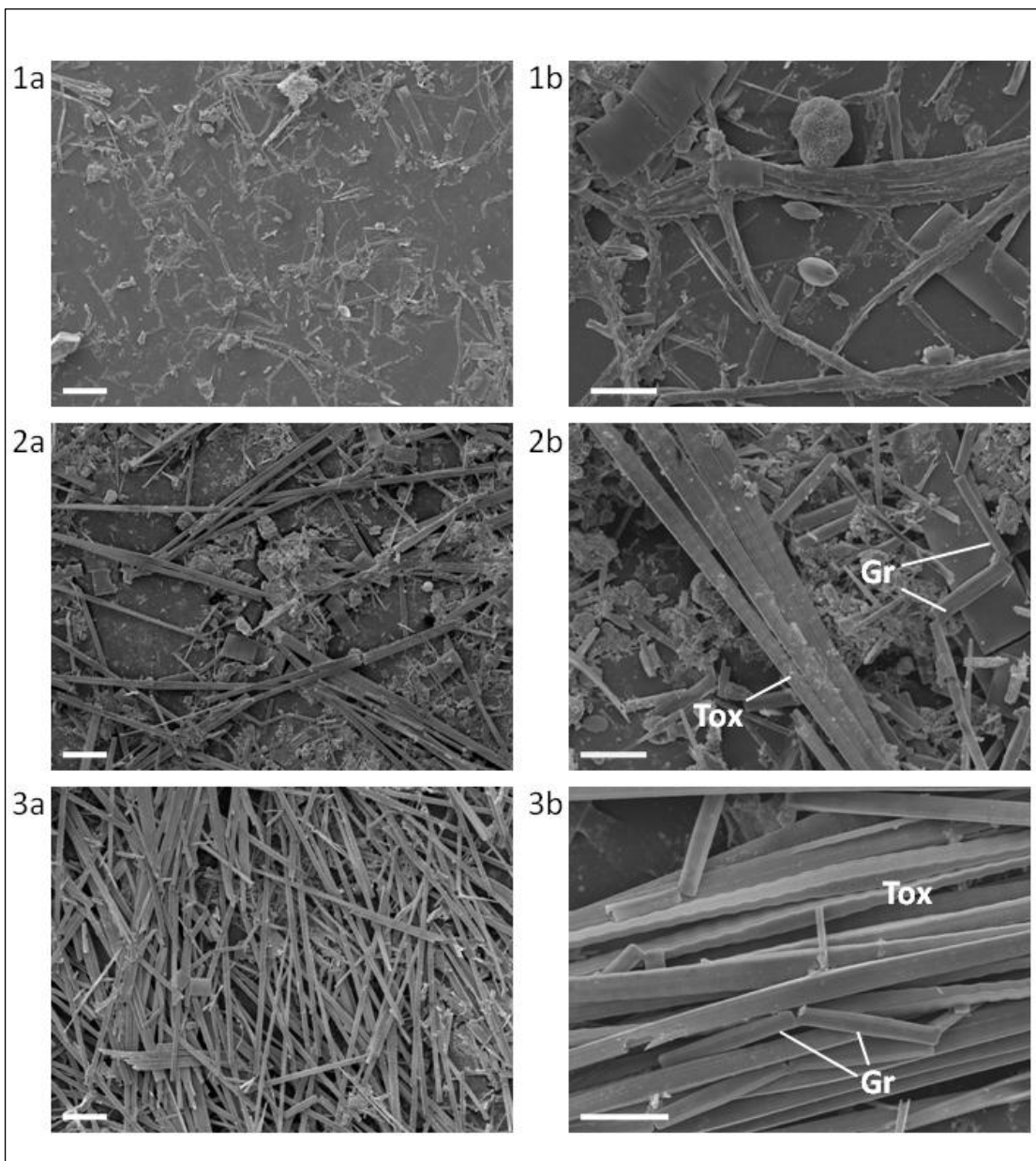


**Fig. 3.4** Hierarchical cluster analysis of the similarity of periphytic diatom assemblage composition based on Ward's method with squared Euclidian distance for all the slides sampled along the Vulcano  $CO_2$  gradient ( $n = 5$  per station). Analysis consists of all genera present over 1 %, including any of the unidentified groups.

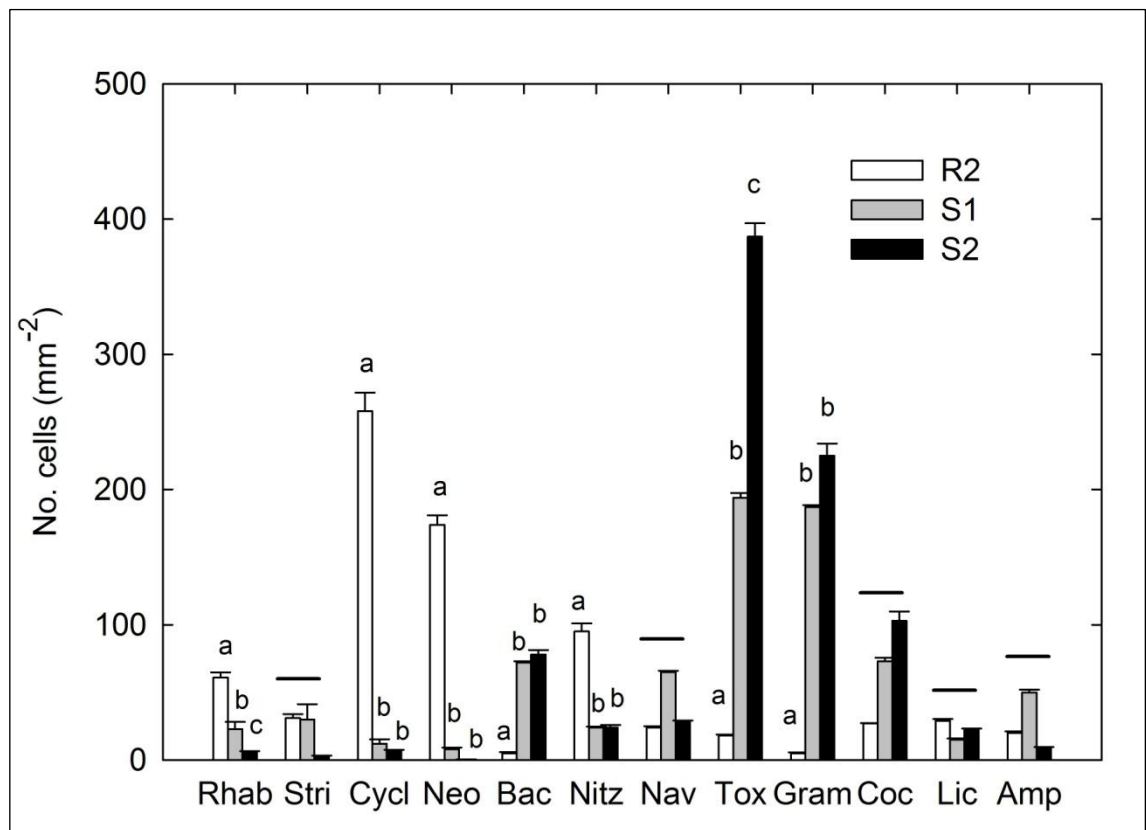


**Fig. 3.5** Relative composition of the periphytic diatom assemblages along the Vulcano CO<sub>2</sub> gradient, including all genera present over 1 % and with all unidentified diatoms grouped into unidentified pennate or naviculoid.





**Fig. 3.6** SEM images of slides colonised by periphytic diatoms at the studied stations along the Vulcano CO<sub>2</sub> gradient; R2 (1a & 1b), S1 (2a & 2b) and S2 (3a & 3b). Diatom colonisation increases with rising  $p\text{CO}_2$  concentrations across the gradient (1a, 2a, 3a). Changes in the community composition also occur with colonies of *Grammatophora* spp. (Gr) and *Toxarium undulatum* (Tox) dominating assemblages in S1 and S2 (2b, 3b) (Scale bars: 1a, 2a, 2b = 100 µm, 1b, 2b, 3b = 50 µm).



**Fig. 3.7** Changes in mean  $\pm$  SE diatom abundances between stations R2, S1 and S2 along the Vulcano CO<sub>2</sub> gradient ( $n = 5$ ). Means with the same letters are not significantly different within each genera across stations ( $P < 0.05$ ), horizontal lines indicate no significant difference ( $P > 0.05$ ).

### 3.4 Discussion

This study presents the first *in situ* assessment of the responses of periphytic assemblages to elevated CO<sub>2</sub> (Johnson et al. 2012a). In order to advance our understanding of how ocean acidification may impact coastal benthic ecosystems it is essential to determine what changes occur at the initial stages of marine community succession as  $p\text{CO}_2$  levels increase. This field study adds to others from CO<sub>2</sub> vent sites that reveal the important biological and ecological changes that are likely to occur as increasing atmospheric CO<sub>2</sub> emissions are absorbed by the surface oceans (Martin et al.

2008; Cigliano et al. 2010; Dias et al. 2010; Porzio et al. 2011, Lombardi et al. 2010; Rodolfo-Metalpa et al. 2010, 2011).

Before the responses of these assemblages are discussed, the increasing trend in silicate concentrations along the Vulcano CO<sub>2</sub> gradient needs to be addressed (see Chapter 2). Silicate is a major limiting nutrient for diatom growth (Martin-Jézéquel et al. 2000) therefore it may be difficult to isolate the effects of elevated CO<sub>2</sub> from elevated silicate. Most of the diatom species that have been examined, however, have a half saturation constant for silicate uptake of ~2 µM and below (Martin-Jézéquel et al. 2000; Claquin et al. 2006). This indicates that silicate uptake rates in diatoms at the reference stations (R1 mean silicate concentration = 3.43 µM) may be near-saturated and that their growth is not therefore limited at these silicate concentrations. Silicate uptake by diatoms between all stations may be similar, despite the increasing trend in silicate concentration along the CO<sub>2</sub> gradient. Furthermore, in oceanic experiments (Egge & Aksnes 1992) diatoms have been found to increase in abundance and dominate phytoplankton assemblages at a threshold silicate concentration of ~ 2 µM. Once this concentration had been exceeded, diatom growth was independent of silica and their abundance remained relatively constant as concentrations were further elevated up to 18 µM (max mean silicate concentration along the gradient, at S3, = 19.39 µM). Therefore it seems reasonable to assume that the differences in silicate concentrations between stations may not be the major driver of change in diatom assemblages when investigating the responses of diatoms along this CO<sub>2</sub> gradient.

The biofilms that developed on the slides from the CO<sub>2</sub> enriched stations were visibly more developed and thicker than those in the reference station. By using Chl *a* as an index of the photosynthetic standing crop, periphyton biomass was found to increase substantially (5 fold) at the CO<sub>2</sub> enriched stations. This indicates that elevations in CO<sub>2</sub>

stimulate primary productivity in these benthic assemblages. The biomass of phototrophic biofilms is known to be regulated by grazing gastropods (Hill & Hawkins 1991; Mak & Williams 1999; Stafford & Davies 2005), populations of which have been found to decrease at low pH at other CO<sub>2</sub> vent systems (Hall-Spencer et al. 2008). The design of the experimental slides in this study, however, eliminates this as a potential confounding factor as the floats to which they were attached were suspended above the benthos (via thin nylon wire), out of the influence of macro-invertebrate grazing. However, these biofilms may still be influenced by zooplankton grazing and any potential pH induced changes in these populations, although this seems unlikely to be the sole driver of the substantial increases in Chl *a* recorded between R2 and S1, S2 in just 21 days. The self shading effects that can occur within thicker biofilms (McNamara & Hill 2000) may be source of variance in Chl *a* measurements and the reduced light intensity has the potential to induce higher concentrations without the corresponding increase in biomass. This however, is unlikely to be the source of variation here as the epi-fluorescence images, SEM photographs and cell counts clearly show a corresponding increase in the number of diatoms with CO<sub>2</sub>-induced Chl *a* elevations.

Diatom epi-fluorescence increased significantly across all stations with increasing CO<sub>2</sub> concentrations, with a 7-fold difference between the R2 and S2 station. This appears to be attributed to the increase in abundance of large pennate types. A potential methodological shortcoming however must be addressed here, epi-fluorescence measurements, despite providing useful percentage surface cover data may not yield accurate information concerning the vertical density of the biofilm. Visual inspection of biofilms however revealed that they were clearly thicker in the CO<sub>2</sub> enriched stations (Fig. 6). In addition, as pH may vary in different layers of a biofilm, future studies should incorporate the use of pH microelectrodes to measure the potential gradient through the microalgal layers. It is also important to consider the potential internal

buffering capacity within these assemblages, i.e. as a result of the high pH generated in dense photoautotrophic biofilms, which may reduce the potential negative effects of low pH on the growth of microalgal cells (e.g. Hama et al. 2012).

Laboratory studies have revealed that pH can affect the adhesion of diatoms to hard surfaces, however, attachment is reportedly favoured in more alkaline conditions greater than pH 7 (Sekar et al. 2004). The greater abundance of diatoms at S1 and S2 is therefore unlikely to be the result of pH-induced preferential attachment. This findings contradict the results of some previous experiments on benthic and planktonic diatom species that suggest their responses to ocean pH changes will be negative (Torstensson et al. 2011) or positive, but small (Tortell et al. 1997; Burkhardt et al. 1999; Crawford et al. 2011). This data supports the notion that some diatoms will benefit from increasing CO<sub>2</sub> through a reduction in the energetic costs of their CCM, optimising resource allocation (Beardall & Giordano 2002; Rost et al. 2008; Trimborn et al. 2009; Hopkinson et al. 2011). These results are also in agreement with laboratory and field incubations that showed CO<sub>2</sub> stimulated the growth of oceanic diatoms (Tortell et al. 2002, 2008; Sun et al. 2011) and complement a recent biofilm laboratory experiment, the findings of which indicated a shift towards diatom dominated biofilms in high CO<sub>2</sub> treatments (Witt et al. 2011). The epi-fluorescence findings indicate that periphytic diatoms, rather than cyanobacteria, were responsible for the differences measured in Chl *a* concentrations and strongly indicate that CO<sub>2</sub> enrichment may stimulate the growth of temperate benthic diatoms. This may have considerable economic repercussions within the shipping industry as diatom dominated biofilms on a ship's hull can increase weight and hydrodynamic drag, increasing fuel costs by up to 15 % (Schultz 2007).

Experimental studies of bloom-forming cyanobacteria usually show a positive response to CO<sub>2</sub> enrichment (Barcelos e Ramos et al. 2007; Hutchins et al. 2007; Levitan et al.

2007; Fu et al. 2008b; Kranz et al. 2009). The findings of this study do not reflect this response as cyanobacteria coverage on the slides remained low (< 2 %) at each station. This is consistent with reports of highly efficient CCM in cyanobacteria (Badger & Price 2002). A similar neutral response to elevated CO<sub>2</sub> has also been observed in endolithic cyanobacteria (Tribollet et al. 2006a). Saturation under ambient pCO<sub>2</sub>, attributed to CCM activity or oligotrophic conditions limiting production was thought to underlie this response and both these reasons may also apply to the Mediterranean cyanobacteria communities of this present study. It can be assumed that the reduction in pH at the CO<sub>2</sub> enriched stations does not limit cell attachment as several laboratory studies have shown that ~ pH 7 creates favourable conditions for cyanobacteria adhesion (Stanley 1983; Vanhaecke et al. 1990; Matsumoto et al. 2000).

The majority of the diatom genera that colonised the slides were the attached forms, araphid and monoraphid groups (e.g *Cocconeis*, *Amphora*, *Toxarium*, *Grammatophora*, *Cyclophora*), however free-living motile forms, pennate biraphids, were also present within the biofilms (e.g *Nitzschia*, *Navicula*). SEM analysis of the composition of diatom populations revealed two contrasting assemblages between the reference site and the CO<sub>2</sub> enriched stations. The changes in carbonate chemistry caused some populations to increase whilst others decreased. Whilst this indicates a shift in competitive outcomes and assemblage structure it only applies for the genera identified; species specific changes in these populations need to be investigated further. The observed increase in the populations of the genera *Toxarium* and *Cocconeis* with CO<sub>2</sub> enrichment presents another potential problem for the shipping industry. These are among the 8-10 genera of diatoms commonly documented as problematic biofoulers of artificial surfaces that have been treated with anti-fouling coatings and the environmentally less damaging alternative, fouling-release coatings (Molino & Wetherbee 2008).

Diversity was significantly reduced at S2. As CO<sub>2</sub> concentrations increased, large and chain forming pennates (*Toxarium* and *Grammatophora*) began to dominate the periphyton assemblages. Similar results have also been found in Southern Ocean phytoplankton assemblages where elevated CO<sub>2</sub> conditions promoted a shift to larger chain-forming *Chaetoceros* spp. (Tortell et al. 2008). The authors attributed this to differences in surface area to volume ratios between genera which would influence competitive outcomes under increasing CO<sub>2</sub> concentrations. Diffusion limitation is thought to become increasingly important as cell size increases (Chisholm 1992; Kiørboe 1993). The exchange of ions between the cell surface and seawater is moderated by the diffusive boundary layer, the thickness of which increases with cell size or aggregate (loose colonies) (Flynn et al. 2012). Under present CO<sub>2</sub> conditions, carbon uptake in smaller species may be maximised by high surface area to volume ratio and a small or absent diffusive boundary layer whilst larger species (which have smaller surface area to volume ratios and a thicker diffusive boundary layer) may be at a competitive disadvantage. This may explain the proliferation of larger, chain-forming taxa under elevated CO<sub>2</sub>. As taxon-specific differences in organism size (and the diffusive boundary layer), metabolic activity and growth rates produce very different microenvironments around the cell, ocean acidification is likely to have varying impacts on plankton physiology (Flynn et al. 2012).

These findings indicate that periphytic diatoms may exhibit non-uniform responses to ocean acidification most likely due to taxon-specific differences in their sensitivity to CO<sub>2</sub> enrichment and presumably due to their kinetics of carbon use. Carbon dioxide / pH induced community shifts have also been observed in many other photoautotrophic assemblages under experimental manipulation (Tortell et al. 2002, 2008; Rost et al. 2003; Fu et al. 2007; Feng et al. 2009; Russell et al. 2009; Trimborn et al. 2009; Connell & Russell 2010; Porzio et al. 2011) adding to evidence that ocean acidification is likely

to lead to structural and functional changes in a wide variety of marine and coastal systems. The establishment of microalgal assemblages on artificial substrata is a complex process. Differences in seasonal recolonisation and succession events (Anderson 1995; Munda 2005) and artificial substratum type (Tuchman & Blinn 1979; Edyvean et al. 1985; Sekar et al. 2004) play an important part in determining the final periphyton assemblage. The diatom composition data presented in this study therefore provides an indication of elevated CO<sub>2</sub> effects on biofilm assemblages rather than a precise analogue for the future effects of CO<sub>2</sub> enrichment. Similar studies should be repeated seasonally, using a variety of substrata and based across different CO<sub>2</sub> gradients to better constrain the large-scale changes that we can expect in response to increases in CO<sub>2</sub> emissions.

The predictions made here are limited to the responses observed from just three stations and therefore three different pH/CO<sub>2</sub> conditions within the same CO<sub>2</sub> vent system. Future experimentation should consider including more than one reference station ensuring patterns observed at these stations are indeed representative of ambient conditions, and also attempt to include more stations within the vent activity region, to extend the range of pH/CO<sub>2</sub> exposure (both of these issues have been addressed in Chapters 4-6). Additionally, logistical constraints (time and financial) limited the extent of the microalgal sampling, specifically the surface area of the biofilms sampled. Future research should aim to increase sampling areas across the biofilms in order to account for the 'patchiness' inherent in natural benthic microalgae assemblages.

The periphyton assemblages analysed here showed significant changes that related to CO<sub>2</sub> enrichment. Increasing CO<sub>2</sub> can stimulate the growth of some benthic diatom species, particularly large, chain forming genera, promoting the primary productivity in marine ecosystems. Therefore, the results of these *in situ* observations (although limited



on temporal and spatial scales) indicate that ocean acidification may have wide ranging consequences from local scale influences on the structure of overlying benthic communities to effects on food web structure and larger scale biogeochemical cycles. It also has the potential to have widespread economic repercussions within marine industries as biofouling rates increase.

## **Chapter 4:** Natural Marine Microphytobenthic Assemblages at Ambient and Elevated Levels of CO<sub>2</sub>

Microphytobenthic assemblages on natural substrata in shallow sub-tidal habitats were investigated along a CO<sub>2</sub> gradient off Vulcano Island, Sicily. The aims of this chapter were to compare epilithic biofilms and microalgal assemblages associated with sandy sediments at normal (present day) and elevated levels of CO<sub>2</sub>. Both microphytobenthic assemblages altered with increasing CO<sub>2</sub>. The photosynthetic standing crop (Chl *a*) in both assemblages increased significantly under conditions of elevated CO<sub>2</sub> indicating that the productivity of shallow water temperate coastal ecosystems could increase under the predicted ocean acidification scenarios. Analysis of the diatom component of the microphytobenthic assemblages revealed that rising CO<sub>2</sub> levels can significantly enhance the growth of benthic diatom populations. There was also a significant shift in the composition of benthic diatom assemblages as CO<sub>2</sub> increased, presumably due to a relative increase in CO<sub>2</sub> tolerant taxa. The abundance of cyanobacteria in surface sediments increased only at extremely high levels of CO<sub>2</sub> (>1,400 µatm), below which abundance was unaffected by increasing levels. The responses of these microphytobenthic assemblages imply that rising CO<sub>2</sub> emissions are likely to have profound ecological and biogeochemical consequences for coastal systems.

## 4.1 Introduction

The term microphytobenthos refers to assemblages of microalgae (Bacillariophyceae, Chlorophyceae, Cyanobacteria and Dinophyceae) and photosynthetic bacteria that colonise benthic substrata. This group is a crucial element of benthic ecosystem functioning (MacIntyre et al. 1996), and therefore any changes in biomass and composition are likely to have reverberating effects throughout the benthos and overlying pelagic systems. This chapter focuses on two different types of microphytobenthic assemblages; those that grow on rock surfaces (epilithic biofilms) and those that colonise submerged sandy sediments. Despite the advantage of being highly standardised, the use of artificial substrata affects the attachment of colonising benthic microflora (Snoeijs 1991) and does not accurately represent natural communities. Sampling communities that have colonised natural surfaces along CO<sub>2</sub> gradients may, therefore, provide more realistic estimates of change in shallow sub-tidal rocky habitats than those obtained by studies using artificial substrata (Cigliano et al. 2010; Johnson et al. 2012a; Lidbury et al. 2012).

Photoautotrophic epilithic biofilms are composed of a variety of unicellular microalgae; on temperate shores they are dominated by benthic diatoms with patches of cyanobacteria (Thompson et al. 2004), whilst in the tropics they are predominately composed of cyanobacteria (Nagarkar et al. 2004). Benthic diatoms are also often the major component of the microphytobenthos on surface sediments where they can constitute more than 90 % of the microalgal cells (Cahoon & Laws 1993). They have been categorised into two groups: epipsammic diatoms which strongly adhere to sediment particles through mucilaginous pads or stalks (Round 1965), and epipellic diatoms which actively move through the sediment via mucilaginous secretion of their raphes (Round 1965, 1971). Cyanobacteria may also be abundant in the

microphytobenthos of sediments and possess the ability to form microbial mats, although this is more likely to occur in extreme environments under which cyanobacteria are released from the control of grazers (Stal 1995).

Photoautotrophic epilithic biofilms are an important functional component of rocky intertidal and shallow sub-tidal habitats around the world (Hill & Hawkins 1991; Bustamante et al. 1995; Jenkins et al. 2001). They are a carbon source for marine trophic webs (Nagarkar et al. 2004; Doi et al. 2008), providing a major food resource for a variety of grazers (Hawkins et al. 1989; Hill & Hawkins 1991; Jenkins et al. 2001) and contributing significantly to primary productivity (Hawkins et al. 1992; Bustamante et al. 1995). They also play important roles in biogeochemical processes (Magalhães et al. 2003) and in the settlement of invertebrates and macro algae propagules (Meadows & Williams 1963; Huang & Boney 1984; Wahl 1989; Thompson et al. 1998).

Sediment microphytobenthic assemblages can also contribute significantly to primary production in both temperate and tropical shallow coastal ecosystems (Colijin & de Jonge 1984; Daehnick et al. 1992; Pinckney & Zingmark 1993; Yallop et al. 1994; MacIntyre et al. 1996; Cahoon et al. 1999; Nelson et al. 1999; Underwood & Kromkamp 1999). Benthic microalgae are often  $10^4$  -  $10^5$  times more concentrated in sediments than in the water (Cahoon 1999). They form an important component of marine food webs providing a primary source of fixed carbon (Heip et al. 1995; Underwood & Kromkamp 1999) and a food supply for a variety of benthic invertebrates (Admiraal 1983; Montagna 1984; Plante-Cuny & Plante 1986; Herman et al. 2000; Huxham et al. 2006). The microphytobenthos can also mediate a variety of biogeochemical processes. At the sediment water interface, horizontal and vertical layers of these assemblages can play important roles in the flux of nutrients and oxygen between benthic and pelagic components of marine systems (Asmus 1984; Wiltshire et

al. 1996; Middleboe et al. 1998; Dong et al. 2000; Lucas et al. 2000; Thorton et al. 2002). Epipellic assemblages have also been found to protect sediments from erosion through the production of sediment stabilising mucilage known as extracellular polymeric substances (EPS) (Holland et al. 1974; Underwood & Paterson 1993a,b; Krumbein et al. 1994; Paterson et al. 1994; Yallop et al. 1994; Noffke 1998; Underwood & Paterson 2003).

The spatial and temporal variability of epilithic biofilms can be influenced by several physical and biological factors such as shore zonation (Castenholz 1963; Christofolletti et al. 2011), season (Underwood 1984a; Hill & Hawkins 1991) and grazing (Stafford & Davies 2005; Skov et al. 2010). Several physical and biological factors are also known to control the biomass and assemblage composition of the microphytobenthos in sediments. Light, temperature, salinity and nutrients can be major factors accounting for variability in microphytobenthic assemblages (Oppenheim 1988; MacIntyre 1996; Underwood et al. 1998; Defew et al. 2004; Van der Grinten et al. 2004, 2005). Some studies have also shown that seasonality can cause patterns in microphytobenthic biomass (Colijn & Dijkema 1981; Colijn & de Jonge 1984; Santos et al. 1997, Sundbäck et al. 2000). Additionally, the feeding activity of benthic meiofaunal grazers can also affect the biomass and distribution of the microphytobenthos (Smith et al. 1996; Sundbäck et al. 1996). Several variables associated with the physical characteristics of the sediment can also affect the microphytobenthos. These include sediment type and grain size (Shaffer & Onuf 1983; Cahoon et al. 1999; Watermann et al. 1999; Jesus et al. 2009), composition (Colijn & Dijkema 1981; de Jong & de Jonge 1995), physical sorting (de Jonge 1985) and stability (Delgado et al. 1991a). Microphytobenthic assemblages at the surface layer of sediments may be disturbed by turbulence and sheer stress generated by tidal currents and wind waves, causing the re-

suspension of cells and loss of biomass (Baillie & Welsh 1980; De Jonge 1985; De Jonge & Van de Bergs 1987; Delgado et al. 1991b; Kendrick et al. 1996).

Recent studies on the impacts of elevated CO<sub>2</sub> on benthic macroalgal assemblages have revealed that there is likely to be decreases in species diversity and profound shifts in community composition (Hall-Spencer et al. 2008; Russell et al. 2009; Fabricius et al. 2011; Porzio et al. 2011). In contrast, the potential consequences of CO<sub>2</sub> enrichment on microphytobenthic assemblages have been relatively overlooked despite their ecological significance. The effects of CO<sub>2</sub> enrichment on the microphytobenthos, have not yet been studied *in situ*. Our current knowledge is limited to a few short term laboratory / mesocosm experiments (Hicks et al. 2011; Witt et al. 2011). In this chapter the responses of shallow, sub-tidal epilithic biofilms and the microphytobenthos associated with sandy sediments (both epipelagic and epipsammic components) to elevated CO<sub>2</sub> were investigated along a CO<sub>2</sub> gradient off Vulcano Island, NE Sicily. The aim was to determine any changes in microphytobenthic biomass and composition, to inform predictions of the impact of CO<sub>2</sub> enrichment in shallow coastal systems.

## **4.2 Methodology**

### **4.2.1 Study sites and carbonate chemistry**

Epilithic biofilms were sampled between 17<sup>th</sup> September to 5<sup>th</sup> October 2010 at stations R1-R3 and S1-S3 along a CO<sub>2</sub> gradient off the island of Vulcano, NE Sicily.

The microphytobenthos on sandy sediments was sampled between the 5<sup>th</sup> and 21<sup>st</sup> May 2011 at stations R4, S1 S2 and S3. Reference stations R1-R3 were unsuitable for this part of the study due to the lack of sandy sediment at shallow depths (< 1.5 m).

See Chapter 2 for full site and station descriptions, and the methodology used to assess carbonate chemistry.

#### **4.2.2 *In situ* sampling**

##### *4.2.2.1 Epilithic biofilms*

Epilithic biofilm sampling occurred in a 5 m x 3 m plot at each station along a CO<sub>2</sub> gradient off Vulcano Island. The sampling plots were shallow sub-tidal areas consisting of a rocky benthos comprising large boulders at ~ 0.5 m. Samples were taken haphazardly from rocks in the sampling plots, separated by a gap of least 30 cm. To reduce potentially confounding biological and physical effects, the biofilm samples were removed from standardised positions on rock surfaces (centre surface of relatively flat-topped rocks) and only from surfaces bereft of macroalgae, macrobiota and encrusting algae.

For the majority of the analyses (except for Chl *a* and SEM analysis which were sampled from rock chippings) a biofilm sampling apparatus (designed and created by Oxford University, Earth Sciences Department) was used to standardise the removal of the epilithic layer of microflora from rock surface in each defined sampling plot. This consisted of an acrylic cylinder, sealed at one end and containing a movable brush appendage. Once an adequate seal was created between the open end of the cylinder and a flat rock surface, the brush appendage was twisted continuously for 2 minutes to dislodge biofilm material from a 7 cm<sup>-2</sup> area. This effectively removed the majority of the biofilm material as it produced bare rock surface, resulting in a visible ‘halo’ effect. The suspended material was then collected through an attached 60 ml syringe. To avoid

cross sample contamination the device was thoroughly rinsed between each use and a new brush head attached at each station.

#### *4.2.2.2 Sandy sediment*

Sediment samples were taken at a depth between 0.5-1.5 m from a predefined area of submerged sediment (5 x 3 m) at each station. Sediment cores were collected using an opened ended circular mould (area = 56.7 cm<sup>2</sup>, depth = 3 cm). For each separate analysis, 10 sediment cores were removed, haphazardly, within the sampling area with a gap of at least 30 cm between samples. From these cores, sub samples (area = 0.64 cm<sup>2</sup>) of the (undisturbed) upper surface were taken to a depth of 5 mm (to include all photosynthetic biomass) using mini corers made from modified disposable 2 ml syringes. To account for patchy distributions of the microphytobenthos and to ensure sufficient material for each analysis, five sub-samples from each sediment replicate were pooled together and treated as one sample. To determine sediment characteristics at each station, three replicate 100 g sediment samples were collected from within the sampling area and air dried.

### **4.2.3 Sample Analysis**

#### *4.2.3.1 Chlorophyll a extraction*

The photosynthetic standing stock of epilithic biofilms was assessed by removing (by hammer and chisel) 30 rock chips (~2 cm x 2cm) from the defined sampling plot at each station for chlorophyll extraction. The photosynthetic standing stock of the microphytobenthos on sandy sediment was assessed from the pooled sediment samples ( $n = 10$  per station). Rock chips and sediment samples were immediately frozen (-20



°C) after collection on site and stored at -80 °C in the laboratory until analysis (< 2 weeks).

As chlorophyll is extracted more efficiently from fully hydrated samples (Thompson et al. 1999), rock chips were first rehydrated for 30 minutes in distilled water. Biofilm material was then brushed off the rock surface in order to avoid cross contamination of the chlorophyll from crustose coralline algae which was often highly associated with the biofilms in the reference stations. Photographs of the rock chips were taken to calculate the surface area of the rock surface sampled using the Image J programme. Sediment samples were first lyophilised to remove water content (which can affect absorbance readings) and to improve the extraction efficiency (Hansson 1988). Chlorophyll was extracted from the rock chip biofilm material and sediment samples using 100 % hot ethanol, and the absorbance of each sample at 632, 649, 665, 696 and 750 nm was measured on a spectrophotometer (Cecil CE2011). Three replicate readings were taken at each absorbance to calculate an average value of Chl *a* for each sample. The total amount of Chl *a* was calculated using the quadrichroic equation of Richie (2008) and expressed per unit surface area ( $\mu\text{g cm}^{-2}$ ) for the rock chips and per unit dry weight for the sediment ( $\mu\text{g g}^{-1}$ ).

#### 4.2.3.2 *Diatom densities*

To determine diatom abundance within the biofilm samples (dislodged biofilm material suspended in 60 ml seawater) and sediment (pooled sediment samples in 20 ml seawater), the samples were first preserved with Lugols Iodine solution (Thronsdon 1978) and stored in a cool, dark place until counting. A total of 10 replicate biofilm and pooled sediment samples were removed from each station for this analysis. Lugols

solution has been shown to induce artefacts which have the potential to introduce bias to the analysis of planktonic samples (Stoecker et al. 1994). In the case of diatoms, artefacts take the form of cell shrinkage (Booth 1987). Whilst this would produce inaccurate estimates of cell volume for studies calculating biomass, for experiments such as this (only concerned with cell counts), Lugols solution remains a suitable preservative.

Diatom abundances were determined using a Sedgewick-Rafter plankton counting chamber (McAlice 1971). The 60 ml bottles containing the biofilm material were shaken vigorously (100 inversions) to break up clumps and re-suspend diatoms. The epipellic and epipsammic diatom components of the sediment samples were enumerated separately. The non-attached epipellic were isolated by shaking the sample in filtered seawater and removing the supernatant, a process repeated 5 times. The epipsammic diatoms were removed from the sand grains by sonication, an optimum sonication period of 10 minutes was set to ensure sufficient diatom removal (~ 90 %) with minimum cell damage (Hickman 1969). After sonication the sample was gently shaken to re-suspend the diatoms which were then removed in the supernatant.

A 1 ml aliquot from each sample was then placed into the chamber for counting under low magnification (x 40) on an inverted light microscope. Between 100-200 diatoms were counted across randomly selected columns in the chamber and the number of diatoms per unit surface area ( $\text{cm}^{-2}$ ) was calculated as follows:

$$\text{Diatom cells / mL} = \frac{\text{diatoms counted}}{\text{grid cells counted}} \times 1000$$

$$\text{Diatom cell density (diatoms/cm}^{-2}\text{)} = \frac{\text{diatoms/mL} \times \text{storage volume}}{\text{surface area of benthos sampled}}$$

Three replicate counts were conducted to give an average diatom density for each sample.

#### 4.2.3.3 Epi-fluorescence Microscopy

A confocal laser scanning microscope (CLSM) was used to determine the abundance of cyanobacteria within epilithic biofilms. Examining biofilm communities on their natural substratum (i.e rock chippings) using CLSM was not possible on this occasion due to the nature of Vulcano's coastal geology. The rugose microtopography of the igneous rock made focusing difficult, limiting the full potential of the fluorescence and potentially inducing experimental artefacts. As an alternative, biofilms were removed using the biofilm sampling apparatus described in section 4.2.2.1. Each 60 ml sample of biofilm suspension ( $n = 10$  per station) was filtered on to 0.2  $\mu\text{m}$  cyclopore polycarbonate membranes (Whatman).

A CLSM was also used to determine the abundance of cyanobacteria within the sediment samples. Dead cells and empty diatom valves in the sediment (often the remains of previous epipellic and epipsammic assemblages and those of surrounding epiphytic and planktonic populations which settle on the bottom sediment after death) can lead to a significant source of error when estimating microphytobenthic populations using light microscopy (Eaton & Moss 1966). Therefore, as this technique discriminates against dead cells, diatom epi-fluorescence was also measured as a proxy for diatom abundance. This technique was not useful for examining the epipsammon due to the uneven microtopography of the sand grains making focusing difficult, so only the epipellic component was analysed with this technique.

To ensure that the chlorophyll retained its autofluorescence for examination at a later date, the filtered biofilm samples and pooled sediment samples were fixed in 2.5 % glutaraldehyde (diluted with filtered seawater) for approximately 1 hour in the dark. The filter papers containing the biofilm were then rinsed in distilled water and mounted on microscope slides. The epipelton was separated from the sediment by the process as described in section 4.2.3.2. The supernatant was filtered through 0.2µm filter papers (Whatman) which were then mounted on microscope slides. All slides were stored at -20 °C (in the field < 48 hours) to ensure the chloroplasts retained their autofluorescence for examination at a later date (Booth 1987; Nagarkar & Williams 1997). When longer storage periods were required i.e. between processing of samples in the UK, storage took place at -80 °C (Thompson et al. 1999). The slides were viewed using a BioRad 2000 Radiance CLSM; excitation 488 nm; emission 570-590/70 and 660-700 nm. A total of 30 images were taken (x10 mag) at random locations across the filter papers and the percentage cover of cyanobacteria fluorescence (and epipellic diatoms) was digitally quantified per image using the Image J software (v 1.43, National Institutes of Health, Bethesda, MD, USA). An average percentage cover was then calculated for each sample from the 30 images.

#### *4.2.3.4 SEM Analysis*

The composition of the epilithic and epipellic diatom community was analysed by a scanning electron microscope (SEM). For analysis of the epilithic biofilms, five replicate rock chips (~2 cm x ~2 cm) were removed from each of the following stations; R2, S1, S2 and S3. Fixed rock chips (2.5 % glutaraldehyde, one hour) were air dried (Hill & Hawkins 1990) and coated with gold prior to SEM observation. The pooled

sediment samples ( $n = 6$  per station) were also fixed in 2.5 % glutaraldehyde for one hour and the epipellic fraction isolated as described section 4.2.3.2. The epipellic fraction was filtered from the supernatant onto 0.2  $\mu\text{m}$  cyclopore polycarbonate membranes (Whatman) which were stored in a phosphate buffer solution until analysis. Filter papers were then also air dried and coated with gold prior to observation under the SEM. Between 200-400 cells were counted and identified to genus level (Round et al. 1990) from randomly positioned images on the colonised areas of rock chips / filter papers. The relative composition of diatom genera was then averaged for each station. Diatoms that could not be accurately identified were assigned to numbered groups (i.e unidentified pennate 1, 2, unidentified naviculoid 1).

#### *4.2.3.5 Sediment properties*

The 100 g sediment samples taken at stations R4, S1-S3 were passed through a settling tube (vertical water column; length 2.5 m, diameter 25 cm) in order to determine sediment settling velocities. Settling velocity data was then converted into grain size (Ferguson & Church 2004). The grain size data was analysed using the software GRADISTAT (Blott & Pye 2001) to determine the following sediment properties; grain size distribution, sediment sorting and skewness (Folk & Ward 1957), sediment type and texture for each station.

#### *4.2.4 Statistical Analysis*

Differences in microphytobenthic assemblages between stations were tested using one-way ANOVA and multiple pairwise comparison post hoc tests (Tukey's Test) were performed where differences were significant. Data that failed tests for normality (Shapiro-Wilk) and homogeneity (Levene Median test) were transformed (arc sin and

ln). When transformations were unsuccessful, data was analysed by Kruskal-Wallis one way analysis of variance on ranks and multiple pairwise comparison post hoc tests (Dunn's method). These statistical analyses were performed using SigmaPlot 11.0.

The abundances of diatom genera were used to calculate Shannon diversity (Shannon and Weaver 1949), Pielou's evenness (Pielou 1975) and Simpsons index of dominance (Simpson 1949) for each rock chip/sediment sample from each station. The similarity of community assemblages across the epilithic biofilms (total  $n = 20$ ) and sediment samples (total  $n = 24$ ) was examined by hierarchical cluster analysis using IBM SPSS Statistics 18. Only genera representing over 1 % abundance were included in this analysis including any of the numbered unidentified diatoms groups. Assemblages were clustered using a dissimilarity coefficient (squared Euclidian distance) and Ward's method (minimum variance clustering).

### **4.3 Results**

#### *4.3.1 Sediment properties*

The sediment characteristics at each station are summarised in Table 4.1. All stations shared similar sediment properties and could all be defined as well sorted, gravelly, coarse sand. The grain size was significantly different between stations (ANOVA:  $F_{(3,8)} = 17.903$ ,  $P < 0.001$ ). However, whilst it was significantly larger in R4 in comparison with the other stations (Tukey,  $P < 0.05$ ), no differences in grain size could be detected between the CO<sub>2</sub> enriched stations S1-S3 (Tukey,  $P > 0.05$ ).

**Table 4.1** Sediment properties of sampling stations along the Vulcano CO<sub>2</sub> gradient.

The grain size distribution was used to calculate various statistical parameters (Folk & Ward 1957) and determine physical descriptions (after Folk 1954) of the sediment in the program GRADISTAT (version 8).

Station	Mean grain size (µm)	Sorting (σ)	Skewness (Sk)	Sediment name	Textural group	Sample Type
R4	912	1.76	-0.121	Slightly fine gravelly coarse sand	Slightly gravelly sand	Unimodal, moderately sorted
S1	600	1.61	0.123	Slightly very fine gravelly coarse sand	Slightly gravelly sand	Unimodal, moderately sorted
S2	499	1.56	0.291	Slightly very fine gravelly medium coarse sand	Slightly gravelly sand	Unimodal, moderately well sorted
S3	547	1.38	-0.04	Slightly very fine, gravelly coarse sand	Slightly gravelly sand	Unimodal, well sorted

#### 4.3.2 Photosynthetic standing crop (Chl *a*)

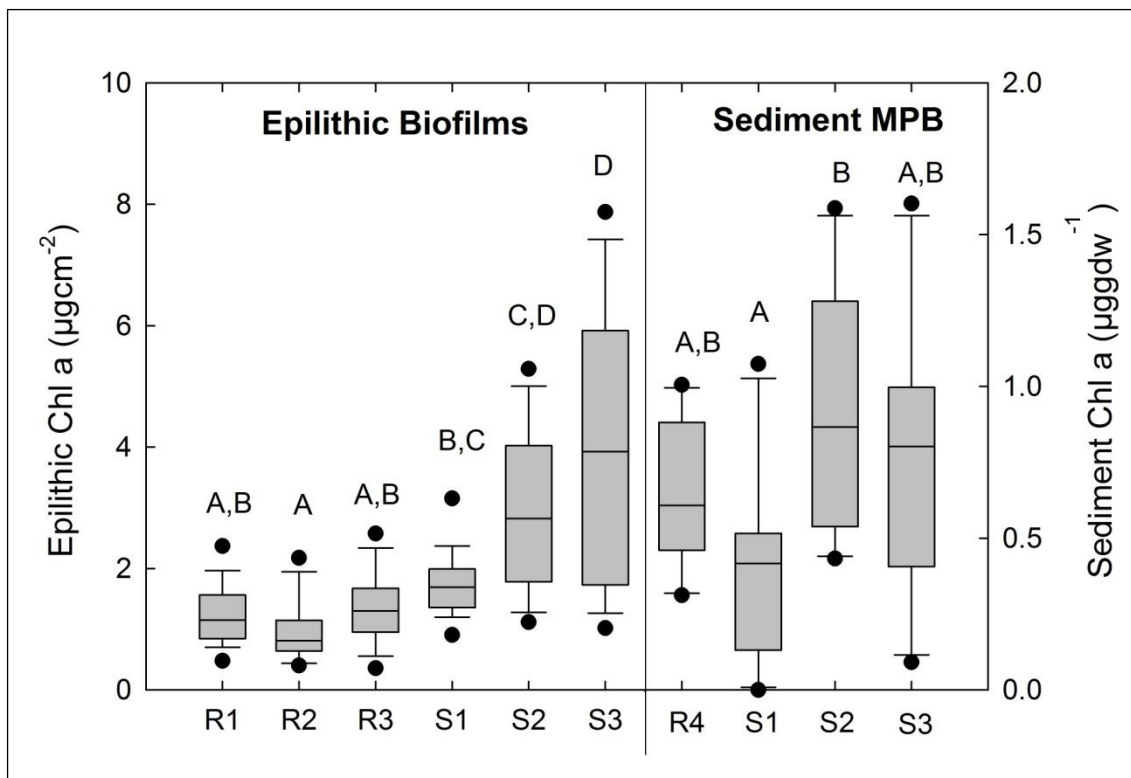
In both epilithic and sediment microphytobenthic assemblages the photosynthetic standing crop (Chl *a*) increased with increasing proximity to the vents (Fig. 4.1). Along the CO<sub>2</sub> gradient significant differences were detected in both epilithic biofilm Chl *a* concentration (Kruskal-Wallis,  $H_5 = 84.219$ ,  $P < 0.001$ ) and Chl *a* content of surface sediment (ANOVA:  $F_{(3,36)} = 3.908$ ,  $P < 0.05$ ). The Chl *a* concentration in epilithic

biofilms was significantly greater in the CO<sub>2</sub> enriched stations S2 (mean Chl *a* = 2.9 µgcm<sup>-2</sup> ± 0.2) and S3 (4.0 µgcm<sup>-2</sup> ± 0.4) compared to the reference stations R1 (1.3 µgcm<sup>-2</sup> ± 0.1), R2 (1.0 µgcm<sup>-2</sup> ± 0.09) and R3 (1.4 µgcm<sup>-2</sup> ± 0.10). No significant differences in epilithic Chl *a* concentrations could be detected between the reference stations R1 and R3 (Dunn, *P*>0.05). In the sandy sediment, the highest mean values of Chl *a* were recorded in the CO<sub>2</sub> enriched stations S2 and S3 (0.92 µg gdw<sup>-1</sup> ± 0.13 & 0.76 µg gdw<sup>-1</sup> ± 0.14, respectively). Post hoc pairwise comparisons however, revealed that Chl *a* content only differed significantly between stations S1 and S2 (Tukey, *P*<0.05).

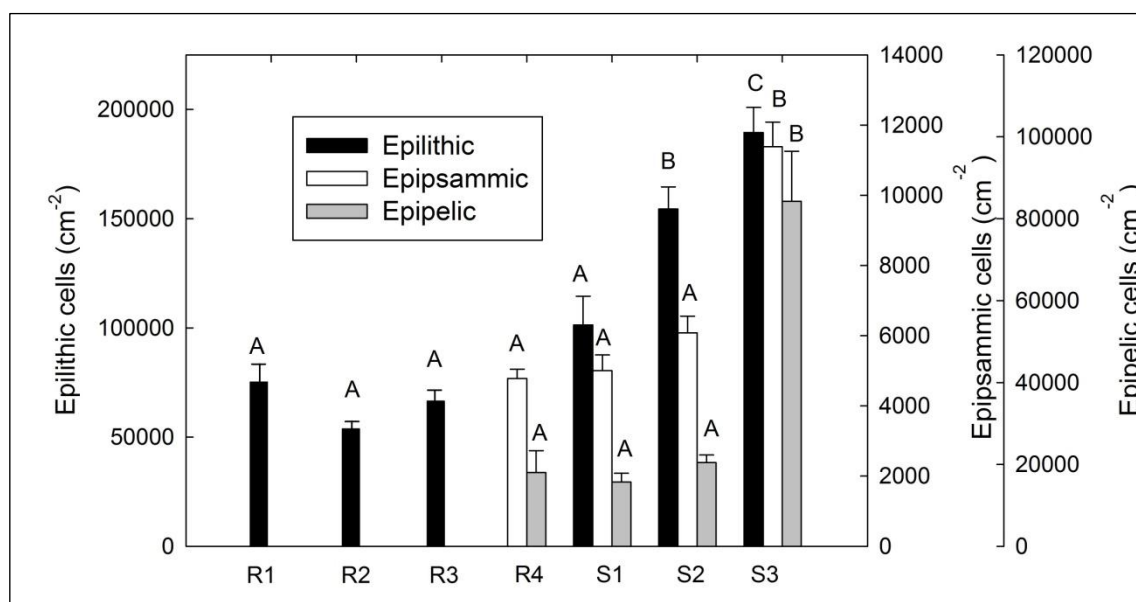
#### 4.3.3 Benthic diatom densities

Benthic diatom densities in both the epilithic biofilms and sediment assemblages increased in the CO<sub>2</sub> enriched stations relative to the reference stations. Along the gradient of increasing CO<sub>2</sub>, significant increases were recorded in the densities of epilithic diatoms (Fig 4.2; ANOVA,  $F_{(5,54)} = 34.554$ , *P*<0.001), epipsammic diatoms (Fig. 4.2; ANOVA:  $F_{(3,36)} = 36.187$ , *P*<0.001) and epipellic diatoms (Fig. 4.2; ANOVA:  $F_{(3,36)} = 24.653$ , *P*<0.001). In the epilithic biofilms the greatest mean densities were found in S3 (189,448 cells/cm<sup>-2</sup> ± 11,494), approximately 3 fold greater than those in the reference stations (R1 = 65,437 cells/cm<sup>-2</sup> ± 5527, R2 = 52,135 ± 4114, R3 = 73,639 ± 7904). Diatom densities in the reference stations did not significantly differ from each other (Tukey *P*>0.05). The densities of the epipsammic and epipellic diatoms were significantly greater in S3 (11,384 cells/cm<sup>2</sup> ± 702 & 84,247 cells/cm<sup>2</sup> ± 12,233, respectively) compared to the remaining stations S2, S1 and R4 (Tukey, *P*<0.05) which were not found to be statistically different from each other (Tukey, *P*>0.05).





**Fig. 4.1** Chl *a* concentration of epilithic biofilms and Chl *a* content of the microphytobenthos (MPB) in surface sediment sampled along the Vulcano CO<sub>2</sub> gradient (median = horizontal line, 25th and 75th percentiles = vertical boxes, 10th and 90th percentiles = whiskers and dots = min/max values, epilithic;  $n = 30$  per station, sediment;  $n = 10$  per station). Boxes with the same letters are not significantly different ( $P > 0.05$ ).

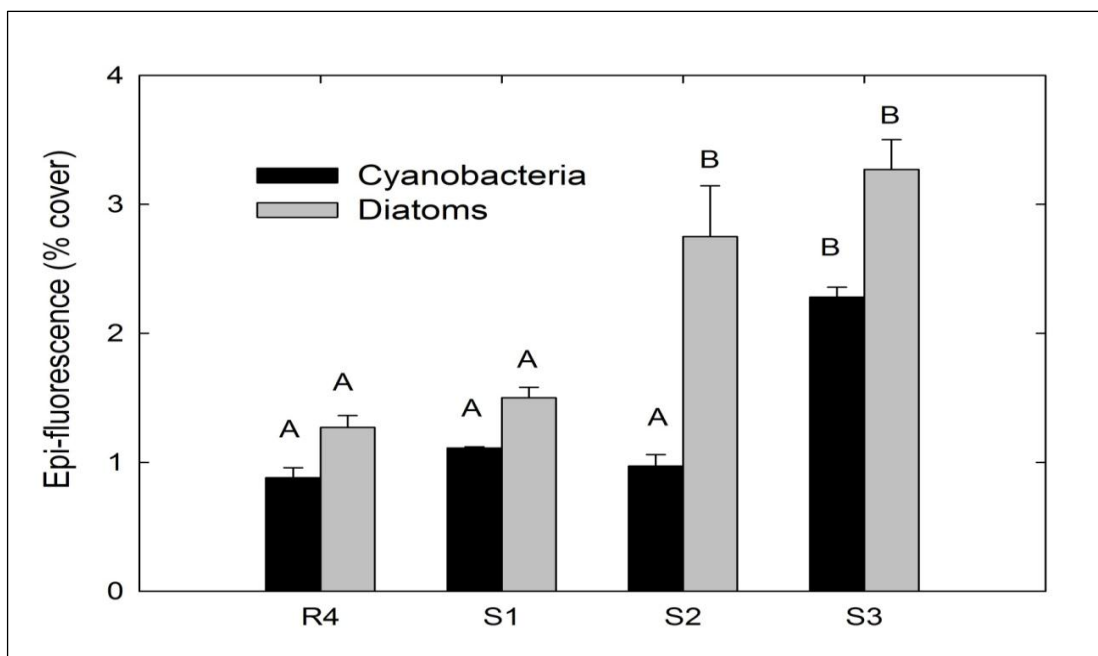


**Fig. 4.2** Mean diatom densities (+ SE) of three benthic assemblages; epilithic, epipsammic and epipelagic sampled along the Vulcano CO<sub>2</sub> gradient ( $n = 10$  per station). Means with the same letters are not significantly different ( $P > 0.05$ ).

#### 4.3.4 Epi-fluorescence analysis

Analysis of the epi-fluorescence images of the epilithic biofilms revealed the presence of filamentous rhodophytes (presumably juvenile stages) inter-dispersed within the collected biofilm material at all stations (Appendix II). Rhodophytes contain the same photosynthetic pigments, phycobilins as cyanobacteria. Therefore, as they both fluoresce at the same wavelength (and can both occur in filamentous, branching forms), these rhodophytes have the potential to mask the presence of any cyanobacteria present within the biofilms and therefore prevent accurate determination of the relative abundances. Analysis of the areal coverage of phycobilin fluorescence however may also serve as a useful indicator of changes in photosynthetic biomass along the CO<sub>2</sub> gradient. Phycobilin fluorescence was the highest at S3 ( $15.4\% \pm 1.8$ ) whilst at stations R1-R3 and S1-S2 the cover ranged between 6-9 %. There was an overall significant

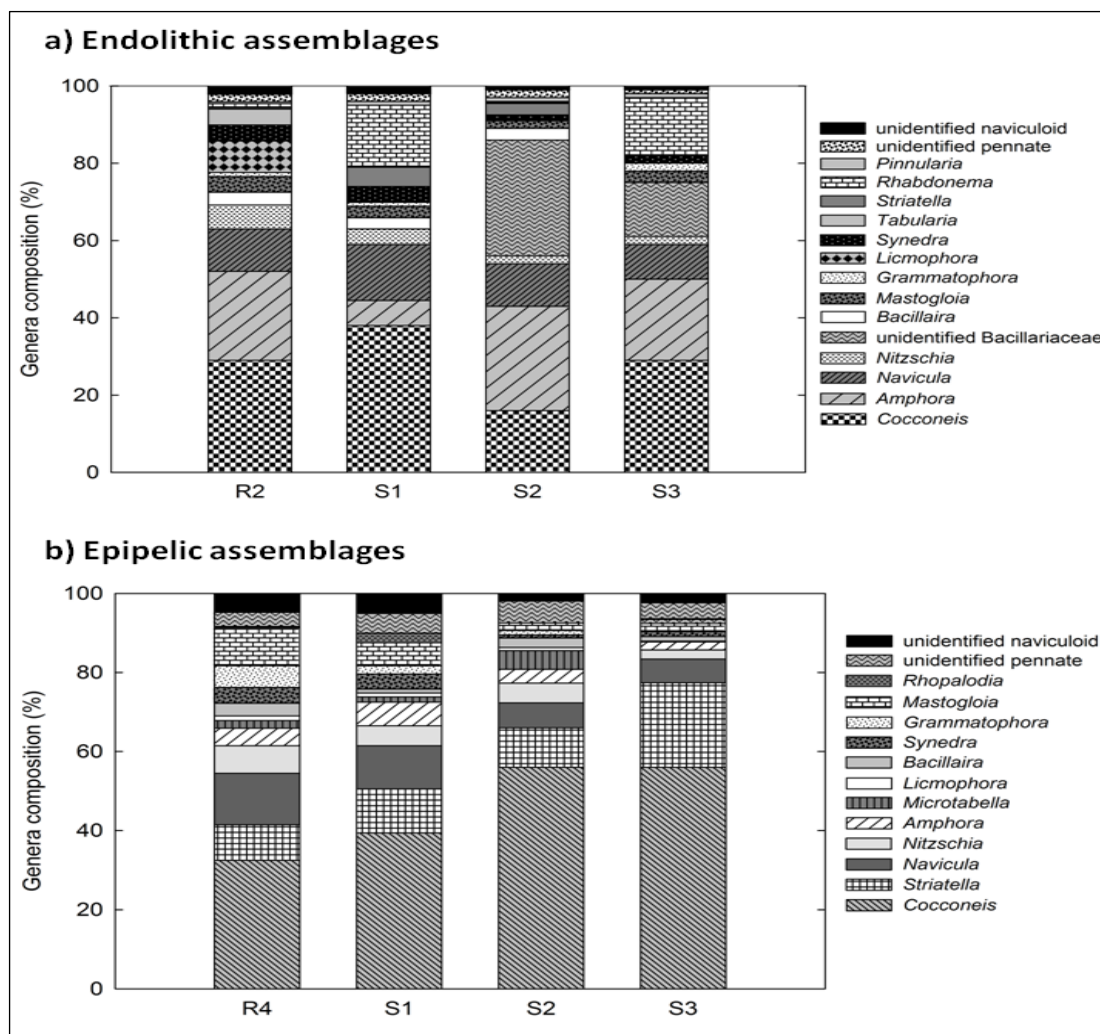
difference detected in the percentage cover of phycobilins within biofilms sampled along the gradient (Appendix II; ANOVA:  $F_{(5,54)} = 9.09$ ,  $P < 0.001$ ), although no significant differences could be detected between R1, R2, R3, S1 and S2 (Tukey,  $P > 0.05$ ). Only the biofilms in S3 had a significantly higher cover of algae containing phycobilins than the remaining stations (Tukey,  $P < 0.001$ ). Epipellic diatom epi-fluorescence was greatest at stations S2 and S3 ( $2.7 \% \pm 0.39$  &  $3.3 \% \pm 0.23$  respectively). It varied significantly between stations (Appendix III, Fig. 4.3; ANOVA:  $F_{(3,36)} = 25.065$ ,  $P < 0.001$ ). The percentage cover of diatom epi-fluorescence was significantly greater at stations S2 and S3 (than R4 (Tukey,  $P < 0.05$ ). The cover of cyanobacteria epi-fluorescence was the greatest at S3 ( $2.3 \% \pm 0.08$ ). It was significantly different along the CO<sub>2</sub> gradient (Appendix III, Fig. 4.3; ANOVA:  $F_{(3,36)} = 52.936$ ,  $P < 0.001$ ). The cover at S3 was significantly greater than at S1, S2 and R4 (Tukey,  $P < 0.05$ ) which were all found to be statistically similar (Tukey,  $P > 0.05$ ).



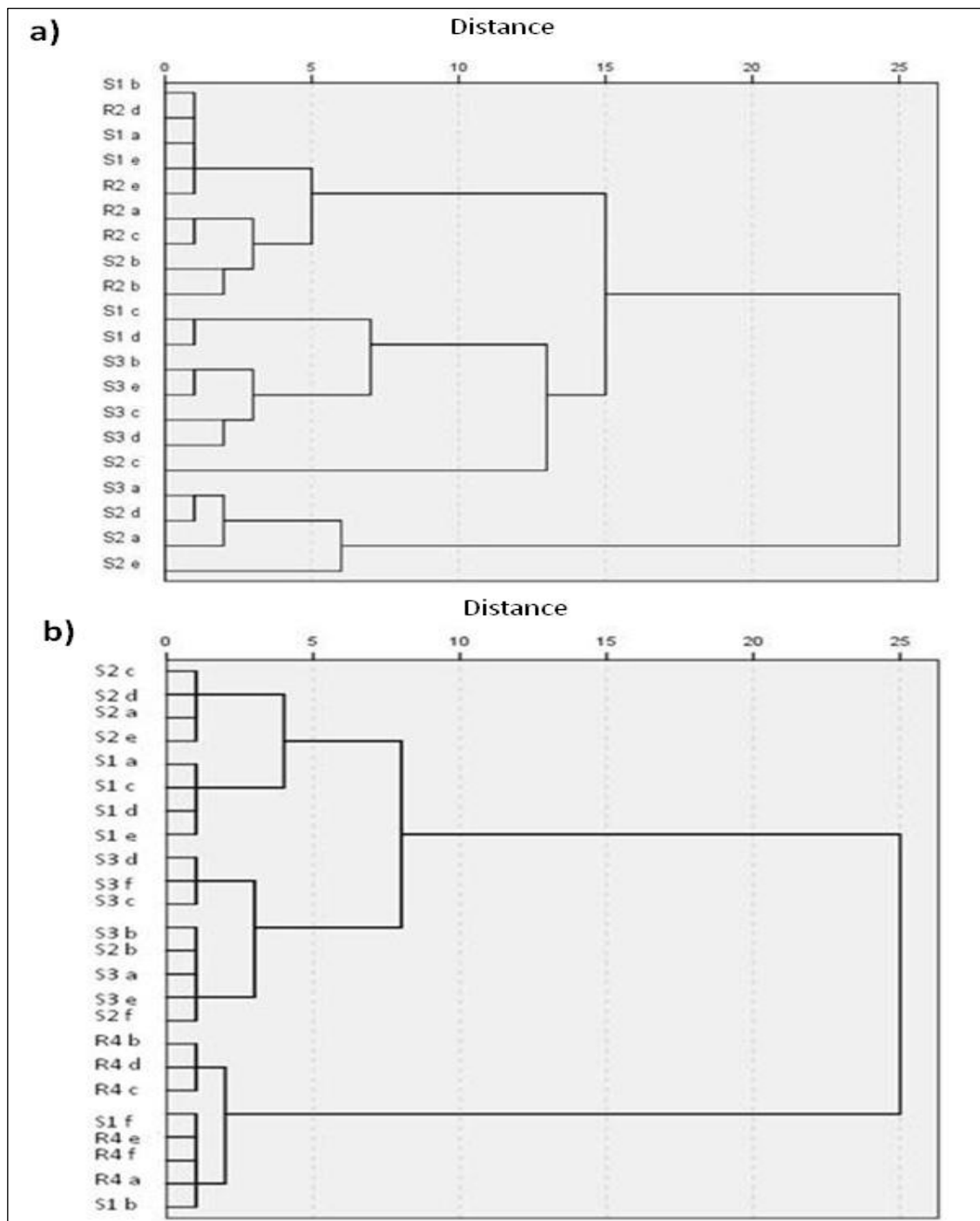
**Fig. 4.3** Mean (+ SE) epi-fluorescence of epipellic diatoms and cyanobacteria sampled from the surface sediment at each station along the Vulcano CO<sub>2</sub> gradient ( $n = 10$ ). Means with the same letters are not significantly different ( $P > 0.05$ ).

#### 4.3.5 Benthic diatom assemblage composition

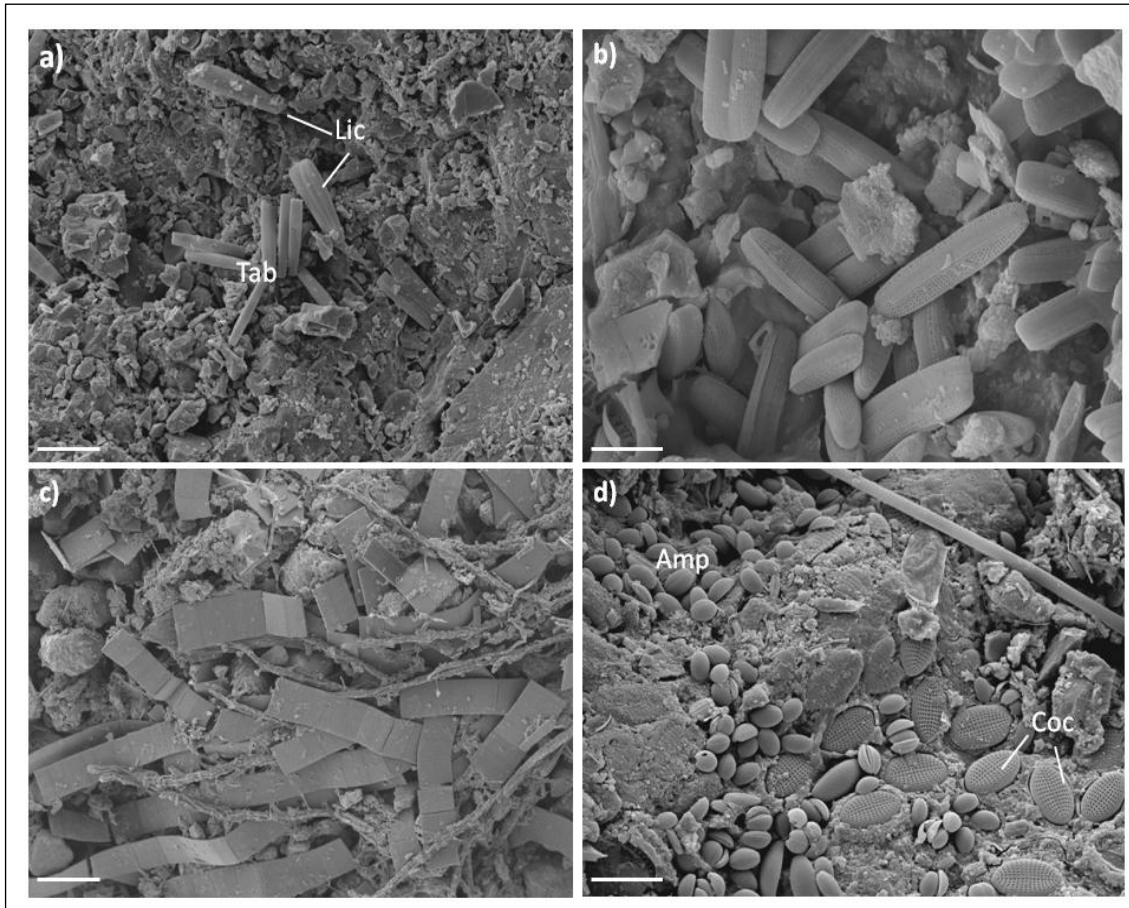
Figure 4.4 shows changes in benthic diatom assemblage compositions along the CO<sub>2</sub> gradient. The diatom composition in the epilithic biofilms sampled from the CO<sub>2</sub> enriched stations S2 and S3 share some similarities and collectively appear to differ from those sampled in R2 (Fig. 4.4a). This is supported by the cluster analysis (Fig. 4.5a) which shows that, overall, assemblages in R2 are widely separated and distinct from those in S2 and S3, although there appear to be some similarities between R2 and S1. Figure 4.6 presents some of the main findings from the SEM analysis of epilithic biofilms. The biofilms sampled in the CO<sub>2</sub> enriched stations (S1-S3) contained a larger proportion of chain forming genera (*Rhabdonema*, *Striatella*, unidentified Bacillariaceae spp.) relative to R2. The biofilms in R2 had a larger fraction of genera that either decreased or were absent in S1-S3 (*Licmophora*, *Tabularia*, *Synedra*). In epipellic assemblages the relative abundance of the genera *Cocconeis* and *Striatella* increased in the high CO<sub>2</sub> stations (S2 & S3) whilst other genera, notably *Mastogloia*, *Grammatophora*, *Synedra*, *Nitzschia* and *Amphora* decreased (Fig 4.4b). The cluster analysis (Fig. 4.5b) revealed that epipellic assemblages in the reference station (R4) were widely separated and distinct from those in the CO<sub>2</sub> enriched stations, particularly S2 and S3.



**Fig. 4.4** Relative composition of epilithic and epipelagic diatom assemblages along the Vulcano CO<sub>2</sub> gradient. Bar charts include all genera present over 1% and all unidentified diatoms grouped as unidentified pennate or naviculoid (epilithic;  $n = 5$  per station, epipelagic;  $n = 6$ ).



**Fig. 4.5** Hierarchical cluster analyses of the similarity of epilithic (a) and epipellic (b) diatom assemblage composition based on Ward's method with squared Euclidian distance for all the biofilms sampled and sediment collected along the Vulcano CO<sub>2</sub> gradient (epilithic  $n = 20$ , epipellic  $n = 24$ ). Analysis consists of all genera present over 1%, including any of the unidentified groups. The epilithic samples from each station ( $n = 5$  per station) are represented by the letters a-e and the epipellic samples from each station ( $n = 6$  per station) are represented by the letters a-f.



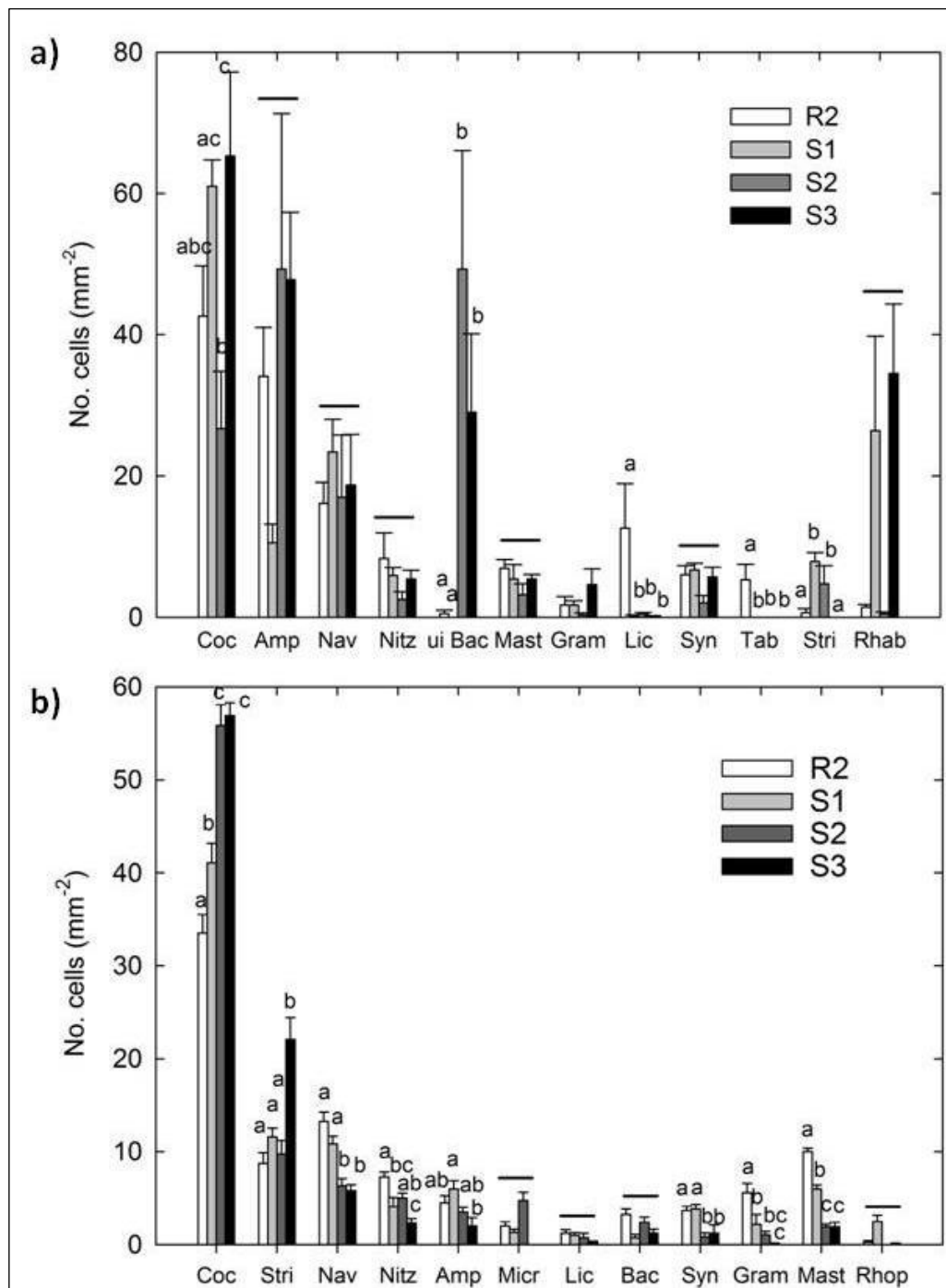
**Fig. 4.6** SEM images of epilithic diatom assemblages.

- a) Patches of *Licmophora* sp. and *Tabularia* sp. attached to rock surfaces by mucilage pads were commonly recorded in the biofilms sampled from R2 (scale bar = 20  $\mu\text{m}$ )
- b) Unidentified Bacillariaceae sp. often found in high densities at stations S2 and S3 (scale bar = 5  $\mu\text{m}$ )
- c) Large colonies of *Rhabdonema* sp. forming ribbon-like chains in high abundances at S1 and S3 (scale bar = 20  $\mu\text{m}$ )
- d) Adnate diatoms such as *Cocconeis* sp. and *Amphora* sp. dominated all biofilms sampled along the  $\text{CO}_2$  gradient (scale bar = 20  $\mu\text{m}$ )

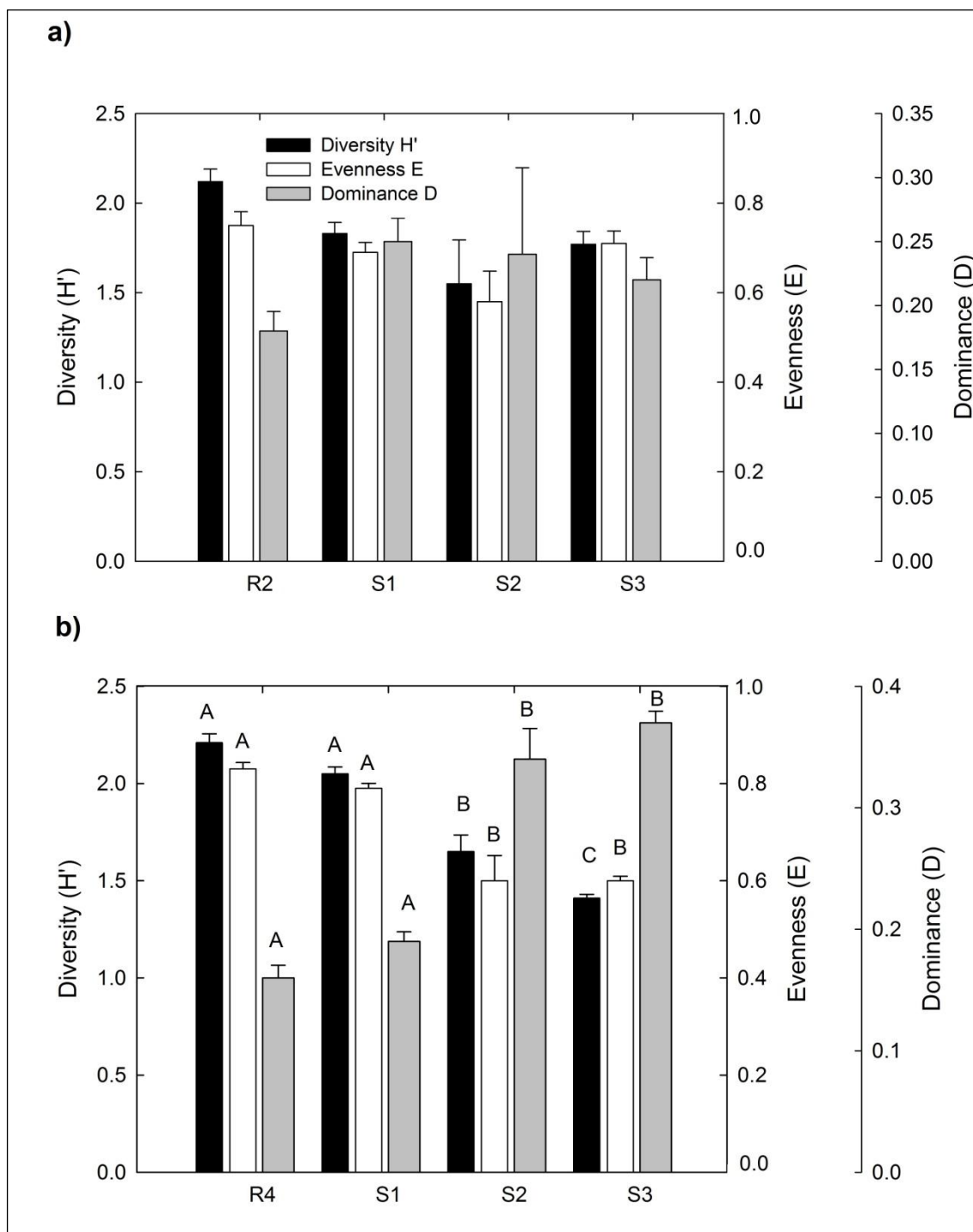
Figure 4.7 displays mean cell counts of the most numerous benthic taxa from the SEM images. It shows that the changes in carbonate chemistry between R2 and S1-S3 relate to the increased abundance of some epilithic genera (*viz.*; *Rhabdonema*, unidentified Bacillariaceae, *Cocconeis*, *Amphora*, *Striatella*, Fig. 4.7a) whilst reducing the abundance of others (*viz.*; *Licmophora*, *Tabularia*). Along the gradient of increasing CO<sub>2</sub> / lowered pH there were significant increases in unidentified Bacillariaceae (ANOVA:  $F_{(3, 16)} = 5.652$ ,  $P < 0.001$ ), *Cocconeis* (ANOVA:  $F_{(3, 16)} = 4.660$ ,  $P < 0.05$ ) and *Striatella* (ANOVA:  $F_{(3, 16)} = 7.264$ ,  $P < 0.001$ ), with significant reductions in *Licmophora* (ANOVA:  $F_{(3, 16)} = 3.814$ ,  $P < 0.05$ ). Some epilithic genera, on the other hand, did not appear to be affected by the changes in pH/CO<sub>2</sub> (*viz.*; *Synedra*, *Mastogloia*, *Navicula*, *Nitzschia*). No significant difference could be detected in the abundances of *Mastogloia* (ANOVA:  $F_{(3, 16)} = 1.103$ ,  $P = 0.377$ ), along the gradient and *Nitzschia* (ANOVA:  $F_{(3, 16)} = 1.415$ ,  $P = 0.275$ ). In epipellic assemblages the changes in carbonate chemistry between R2 and S1-S3 also relate to the increased abundance of some genera (*viz.*; *Cocconeis*, *Striatella*, Fig. 4.7b) whilst reducing the abundance of others (*viz.*; *Navicula*, *Nitzschia*, *Grammatophora*, *Mastogloia*, *Synedra*, *Amphora*). There were significant increases in the abundance of *Cocconeis* (ANOVA:  $F_{(3, 20)} = 36.251$ ,  $P < 0.001$ ) and *Striatella* (ANOVA:  $F_{(3, 20)} = 14.027$ ,  $P < 0.001$ ) along the CO<sub>2</sub> gradient and significant reductions in the abundances of *Navicula* (ANOVA:  $F_{(3, 20)} = 19.425$ ,  $P < 0.001$ ), *Nitzschia* (ANOVA:  $F_{(3, 20)} = 12.219$ ,  $P < 0.001$ ), *Grammatophora* (ANOVA:  $F_{(3, 20)} = 9.013$ ,  $P < 0.001$ ), *Mastogloia* (ANOVA:  $F_{(3, 20)} = 90.032$ ,  $P < 0.001$ ), *Synedra* (ANOVA:  $F_{(3, 20)} = 9.327$ ,  $P < 0.001$ ) and *Amphora* (ANOVA:  $F_{(3, 20)} = 4.690$ ,  $P < 0.001$ ). Some genera, on the other hand, did not appear to be affected by the changes in pH/CO<sub>2</sub> such as *Licmophora* (ANOVA:  $F_{(3, 20)} = 2.203$ ,  $P = 0.119$ ).



The epilithic diversity ( $H'$ ) was the greatest in R2 ( $2.12 \pm 0.07$ ) and decreased in CO<sub>2</sub> enriched stations ( $S1 = 1.83 \pm 0.06$ ,  $S2 = 1.55 \pm 0.24$ ,  $S3 = 1.77 \pm 0.07$ ). There was, however, no significant difference in the generic diversity of epilithic diatoms between the stations (Fig. 4.8a; ANOVA:  $F_{(3,16)} = 2.927$ ,  $P = 0.066$ ). Similarly, no significant differences were detected in the biofilm diatom assemblage evenness ( $J'$ ) (ANOVA:  $F_{(3,16)} = 3.113$ ,  $P = 0.056$ ) or dominance (D) (ANOVA:  $F_{(3,16)} = 0.718$ ,  $P = 0.556$ ) between stations. The generic diversity of epipelagic assemblages also decreased significantly in high CO<sub>2</sub> (Fig. 4.8b; ANOVA,  $F_{(3,20)} = 48.120$ ,  $P < 0.001$ ), whilst the dominance index was significantly higher in S2 and S3 than the other two stations (ANOVA:  $F_{(3,20)} = 47.516$ ,  $P < 0.001$ ; Tukey,  $P < 0.05$ ). Epipelagic evenness decreased in the CO<sub>2</sub> enriched stations. It significantly varied between stations (Fig. 4.8b; ANOVA;  $F_{(3,20)} = 19.877$ ,  $P < 0.001$ ). Station R4 had significantly greater evenness than S2 and S3 (Tukey,  $P < 0.05$ ).



**Fig. 4.7 a)** Changes in mean (+SE) epilitic diatom abundances between stations R2, S1, S2 and S3 ( $n = 5$  per station) along the Vulcano  $\text{CO}_2$  gradient. **b)** Changes in mean (+SE) epipelagic diatom abundances between stations R4, S1, S2 and S3 ( $n = 6$  per station) along the Vulcano  $\text{CO}_2$  gradient. Means with the same letters are not significantly different within each genera across stations ( $P < 0.05$ ), horizontal lines indicate no significant difference ( $P > 0.05$ ).



**Fig. 4.8** Mean (+ SE) values for generic diversity (Shannon  $H'$ ), evenness (Pielou's  $E$ ) and dominance (Simpsons index  $D$ ) of epilithic (a) and epipelagic (b) diatom assemblages along the Vulcano  $CO_2$  gradient (epilithic  $n = 5$  per station, epipelagic  $n = 6$  per station). In graph (a), means are all statistically similar ( $P > 0.05$ ). In graph (b), means with the same letters within each metric across stations are not significantly different ( $P > 0.05$ ).

## 4.4 Discussion

Microphytobenthic assemblages play a crucial role in marine and coastal habitat functioning (MacIntyre 1996; Underwood & Kromkamp 1999; Jenkins et al. 2001; Thornton et al. 2002). The potential impacts of ocean acidification on these assemblages, however, are poorly understood. This study is the first to investigate the *in situ* effects of CO<sub>2</sub> enrichment on both shallow sub-tidal epilithic biofilms and epipellic and epipsammic microalgal assemblages.

### 4.4.1 Epilithic biofilms

Along the CO<sub>2</sub> gradient the photosynthetic standing crop of biofilms was significantly altered. Chl *a* concentrations increased incrementally with rising CO<sub>2</sub> levels and, at the highest exposure in S3 showed a 3-fold increase from the reference stations. This supports the findings from comparable studies into microphytobenthic assemblages on artificial hard surfaces (Lidbury et al. 2012; Johnson et al. 2012a), indicating that elevations in CO<sub>2</sub> can enhance primary productivity in benthic microalgal assemblages. The biomass of epilithic biofilms is known to be regulated by grazing gastropods (Norton et al. 1990; Hill & Hawkins 1991; Mak & Williams 1999; Stafford & Davies 2005) which decrease in abundance due to shell dissolution along the CO<sub>2</sub> vent gradient (Hall-Spencer et al. 2008). Release from grazers may therefore play a role, although comparable increases in Chl *a* with CO<sub>2</sub> enrichment were reported in the periphytic communities studied in Chapter 3 (Johnson et al. 2012a), under experimental conditions which eliminated gastropod grazing as a co-variable. However, as discussed in Chapter 3, zooplankton grazing as a potential co-variable needs to be considered. CO<sub>2</sub>-induced changes in grazing activity do affect algae along the gradient (Johnson et al. 2012b).

The response observed in this study may therefore be a result of the cumulative effects of changes in the carbonate chemistry and release from micro and macro grazers.

Herbivore exclusion experiments would be useful to determine the relative role of CO<sub>2</sub> enrichment and grazing pressure release as drivers of the observed responses to acidified seawater.

Analysis of the epilithic biofilms revealed an increase in the abundance of benthic diatoms with CO<sub>2</sub> enrichment. Abundances at the CO<sub>2</sub> enriched stations S2 and S3 were more than double those found at the reference stations. These findings contradict the results of those laboratory experiments that suggest the diatom responses to CO<sub>2</sub> are negative or small (Tortell et al. 1997; Burkhardt et al. 1999; Crawford et al. 2011; Torstensson et al. 2011) but are in agreement with studies that have documented positive responses of diatoms to elevated CO<sub>2</sub> (Tortell et al. 2002, 2008; Feng et al. 2009; Sun et al. 2011; Gao et al. 2012). It is possible, based on the literature to date (although this is heavily biased towards oceanic microalgae) that benthic diatoms may be benefiting from the increasing CO<sub>2</sub> through a reduction in the energetic costs of their CCM and optimised resource allocation, a notion gaining a considerable amount of acceptance (Beardall & Giordano 2002; Rost et al. 2008; Trimborn et al. 2009; Hopkinson et al. 2011).

This study is limited with regard to predictions concerning the effects of high CO<sub>2</sub> on cyanobacteria in epilithic biofilms due to the presence of filamentous rhodophytes which share the same fluorescence properties and in some cases, the same morphologies as cyanobacteria. The presence of macroalgae (juvenile stages) indicates that the epilithic biofilms in this study are at a more mature stage of development compared to those studied in Chapter 3 (21 days). Phycobilin fluorescence was enhanced in the highest CO<sub>2</sub> station (S3). This indicates that there are some positive effects of CO<sub>2</sub> on

phycobilin containing algae in these biofilms but only under extreme levels of  $p\text{CO}_2$  ( $> 1,400 \mu\text{atm}$ ). The increase in this fluorescence is more likely to be due to rhodophytes which appeared to proliferate in S3 (pers. obs). This is consistent with a laboratory study which observed positive effects of high  $\text{CO}_2$  on the red alga *Lomentaria articulata* (Kübler et al. 1999) and a field study which recorded an increase in abundance of fleshy rhodophytes along a natural volcanic  $\text{CO}_2$  gradient (Porzio et al. 2011). It is, however, more difficult to comment accurately on the potential changes in cyanobacterial abundance, due to the masking effect of the larger filamentous rhodophytes.

The benthic diatom assemblage composition altered under  $\text{CO}_2$  enrichment indicating genera specific differences in diatom sensitivity to  $\text{CO}_2$  concentrations, consistent with the findings from Johnson et al. (2012a). This may be attributable to the differences in carbon acquisition abilities that exist between taxa (Tortell 2000; Burkhardt et al. 2001; Giordano et al. 2005) and supports the findings of numerous other studies which have observed genera specific responses of both oceanic and benthic diatoms to changing  $\text{CO}_2$  levels (Tortell et al. 2002, 2008; Rost et al. 2003, Fu et al. 2007; Feng et al. 2009; Trimborn et al. 2009; Johnson et al. 2012a). Species specific interactions between benthic diatoms and settling macroalgal sporelings are known to exist (Huang & Boney 1984, 1985) and have the potential to determine the outcomes of early colonisation events on surfaces in the marine environment. Therefore, changes in the relative composition of different diatom genera in epilithic biofilms may have wider reaching ecological consequences, with the potential to alter the structure of overlying benthic communities.

The genera *Amphora*, *Cocconeis* and *Navicula* in epilithic biofilms remained relatively constant along the gradient, however abundances of other genera appeared to vary with  $\text{CO}_2$  enrichment. The relative abundance of some genera decreased with  $\text{CO}_2$

enrichment (*Licmophora*, *Tabularia*, *Synedra*), whilst there was an increase in the chain forming genera, *Rhabdonema*, *Striatella* and an unidentified genera from the family Bacillariaceae. This is consistent with the findings from Chapter 3 (Johnson et al. 2012a) which reported increases in the dominance of large chain forming periphytic *Grammatophora* and *Toxarium* with CO<sub>2</sub> enrichment and from a study of phytoplankton assemblages which observed a shift in dominance to chain forming *Chaetoceros* spp. (Tortell et al. 2008). Collectively, these findings strengthen support for the idea that elevated CO<sub>2</sub> has the ability to influence the competitive abilities amongst different size classes / morphologies of diatoms, causing shifts in the structures of diatom assemblages in both benthic and pelagic habitats. This may be attributed to differences in CO<sub>2</sub> diffusion limitation between various diatom size groups and morphologies (Chisholm 1992; Kiørboe 1993).

Diatom assemblages from the biofilms at the reference station were significantly dissimilar to the majority of those sampled in the intermediate and high CO<sub>2</sub> stations (S2 and S3, respectively). There was a greater variation in assemblages between the CO<sub>2</sub> enriched stations relative to those observed on the artificial surfaces (perspex) studied in Chapter 3. The natural rock surfaces studied in this chapter have a more variable microtopography compared to the smooth substrata used in Chapter 3. Differences in surface rugosity influence microalgal colonisation (Thompson et al. 1999), and the micro-texture of surfaces has affects on the composition of biofilms (Edyvean & Moss 1985; Faimali et al. 2004). Despite restricting sampling to relatively flat sub-tidal rock surfaces, potential variability in the surface micro-texture of the igneous rock chippings may contribute to the greater variation between the assemblages of epilithic diatoms in the CO<sub>2</sub> enriched stations relative to the more standardised artificial surfaces. Furthermore, differences in the dominant genera observed between the different substrata under high CO<sub>2</sub> (*Toxarium*, *Grammatophora* on perspex slides

vs *Rhabdonema* and *Striatella* on rock surfaces) is consistent with several other studies that have noted differences in microalgal colonisation between natural and artificial surfaces (Siver 1977; Tuchmann & Blinn 1979; Tuchman & Stevenson 1980; Edyvean & Moss 1985; Woods & Fletcher 1991) and highlights the importance of studying a range of colonisation surfaces before wider scale predictions of the expected changes in benthic diatoms under ocean CO<sub>2</sub> enrichment can be formulated.

#### 4.4.2 *Microphytobenthos of sandy sediments*

Permeable sands are common coastal environments (Riggs et al. 1996) and cover approximately 70 % of the continental shelf (Emery 1968). As a significant portion of these sediments receive sufficient light to sustain primary production (Gattuso et al. 2006) they are considered important contributors to shelf and global carbon cycles (Jahnke 2004; Evard et al. 2008). It is therefore important to determine the responses of microphytobenthic assemblages in sandy sediments to CO<sub>2</sub> enrichment. It should be noted that this part of the study is disadvantaged by the fact there can be no measure of natural variability. This is because only one reference station, R4, was included, but due to the lack of sandy sediment at constant depth along the CO<sub>2</sub> gradient this was unavoidable.

Sediment type is often the source of most variation in microphytobenthic assemblages. Significant differences in biomass, depth distribution, species diversity and assemblage composition have been observed between muddy and sandy sediments (Watermann et al. 1999; Patterson & Hagerthey 2001; Cartaxana et al. 2006; Underwood & Barnett 2006; Jesus et al. 2009). The sediment analysis revealed that the four sampling stations along the CO<sub>2</sub> gradient in this study shared similar characteristics and properties, all sediment samples could be classed as well sorted, gravelly coarse sand. Based on these



findings it is therefore unlikely that the sediment properties between stations are a significant confounding factor in this study, therefore the microphytobenthic responses observed may be attributed to the direct and indirect effects of changing carbonate chemistry along the gradient. It is possible that the pH and nutrients within sediment differs substantially from that measured in the overlying water column. Whilst this may have implications for the conclusions drawn, it does, however, seem unlikely as only the surface sediments (top 5 mm) were sampled in this study. One other important consideration is the potential for pH buffering within sediments. The pH perturbation may have been buffered by alkalinity generation which occurs through the dissolution of carbonate minerals in sediments (Andersson et al. 2007). It is therefore possible that the alkalinity generation may negate high CO<sub>2</sub> perturbation, this may result in different biological responses within sediments compared to those in the water column. This was recently demonstrated in a mesocosm and field study into the impacts of ocean acidification on microbial ammonia oxidation activity (Kitidis et al. 2011). However, the Baia di Levante was created by the last volcanic explosion on the island in 1888, and as a result the volcanic sand is notably depauperate in carbonate (L. Petit; unpublished data). Therefore the sediments present will be a negligible source of alkalinity production.

Sediment Chl *a* content was greater at stations S2 and S3 indicating CO<sub>2</sub> enhancement of primary production in the microphytobenthic assemblages. This is comparable with the responses of the epilithic biofilms and is consistent with the findings from the previous chapter (Chapter 3, Johnson et al. 2012a). These results, however, are not consistent with a recent laboratory experiment on the microphytobenthic assemblages of muddy sediments, which failed to detect any influence of CO<sub>2</sub> alone on microphytobenthic biomass (Hicks et al. 2011). Despite the largest Chl *a* content being recorded in S2 and S3, unlike the epilithic biofilm findings and those from Chapter 3,

there was no concomitant increase in Chl *a* with CO<sub>2</sub>. Potential methodological issues, such as a smaller sample size, and the inherent problems associated with extracting Chl *a* from sediment may explain the relatively poor correlation between levels of CO<sub>2</sub> and Chl *a* in this study. High concentrations of organic matter, humic acids, phenolic compounds, bacteriochlorophylls and photopigment degradation products in the sediment may interfere with Chl *a* estimates using spectrophotometric techniques (Pinckney et al. 1994; R. Ritchie pers. comms). So, any station specific differences in the relative abundance of these compounds may have obscured patterns of incremental increase in Chl *a* with CO<sub>2</sub>.

Epipsammic and epipellic diatoms abundances were considerably greater at the highest CO<sub>2</sub> exposure (S3). The epi-fluorescence measurements of epipellic diatom assemblages also indicate that diatom abundance was the greatest at this station. This confirms that the large increase in the abundance of diatoms at S3 can be mainly attributed to the counting of live cells as opposed to the frustules of dead diatoms in the sediment. Together with the findings from the epilithic assemblages, these results support several other studies which have observed positive responses of diatoms to CO<sub>2</sub> enrichment (Tortell et al. 2002, 2008; Feng et al. 2009; Sun et al. 2011; Johnson et al. 2012a). There was, however, no incremental increase observed in epipellic and epipsammic diatom abundances between the other stations along the gradient, suggesting that the correlation between diatom abundance and CO<sub>2</sub> enrichment is less pronounced within these assemblages compared to those in epilithic biofilms. In contrast however, a concomitant increase in epipellic diatom epi-fluorescence with CO<sub>2</sub> was observed along the gradient. Further research and potentially larger sample sizes may be required to clarify the discrepancies between these two datasets.

The cyanobacterial component of the sediment epipelon increased under extreme CO<sub>2</sub> exposure (S3) only (> 1,400 µatm). There were no measurable effects observed under intermediate-high concentrations of CO<sub>2</sub>, consistent with the periphyton findings from Chapter 3 (Johnson et al. 2012a) and similar to those reported by Tribollet et al. (2006a) which failed to detect a significant effect of CO<sub>2</sub> levels on endolithic assemblages elevated up to 750 ppm. The slight elevations recorded in nutrients at S3 (see Chapter 2) coincide with the sudden proliferation of cyanobacteria at high CO<sub>2</sub>. This suggests that the relatively oligiotrophic conditions along the gradient may limit the potential enhancement of primary production caused by elevated CO<sub>2</sub> (as also may be the case in the experimental tanks in Tribollet et al. 2006a). This response may also be explained, in part, by the high efficiency of cyanobacterial CCM (Badger & Price 2002) causing them to be relatively less sensitive to changing CO<sub>2</sub> than diatoms, for example and only exhibit responses once high concentrations of CO<sub>2</sub> have been reached (i.e > 1,400 µatm). These results contrast with several experimental studies of oceanic bloom forming species (Barcelos e Ramos et al. 2007; Hutchins et al. 2007; Levitan et al. 2007; Fu et al. 2008b; Kranz et al. 2009) which reported significant, positive interactions of elevated CO<sub>2</sub> on photosynthesis, nitrogen fixation and growth. Crucially, however, these experiments appear to have been conducted in nutrient replete conditions which may explain the differences in responses. Therefore, under more eutrophic conditions, we may expect benthic cyanobacteria to respond positively to CO<sub>2</sub> enrichment. Further experimentation, however, is clearly warranted

The majority of the epipellic diatom assemblages analysed consisted of motile biraphid genera (*Navicula*, *Nitzschia*, *Mastogloia*, *Amphora*) typical of these habitats. The most dominant genera in all samples however was, surprisingly, *Cocconeis*, an attached diatom usually associated with the epipsammon and a major component of epilithic and epiphytic assemblages. High frequencies of this diatom have also been observed in

other sediment habitats (Facca & Sfriso 2007) which the authors attributed to the high biomass of macrophytes forming the source of the *Cocconeis* spp. found in epipelon. Due to the sandy nature of the sediment in this study the detached *Cocconeis* cells are most likely derived from the epipsammon and the epi-fluorescence data indicates that they still form a photosynthetically active component of the epipelon, eliminating the possibility of them being empty frustules.

The taxonomic composition of microphytobenthic assemblages is determined by complex interactions between factors such as light, temperature, salinity, nutrients and sediment type (Oppenheim 1988; Underwood 1994; Van der Grinten et al. 2004, 2005; Facca & Sfriso 2007; Jesus et al. 2009). The composition of epipellic diatom assemblages altered significantly along the gradient and, as light, temperature, salinity, nutrients, and sediment type remained relatively constant between stations (see Chapter 2), these changes seem related to the varying levels of CO<sub>2</sub>. As *p*CO<sub>2</sub> levels increased some genera increased in abundance, whilst others decreased. As discussed with the epilithic biofilm findings, this indicates a CO<sub>2</sub> induced shift in competitive outcomes and assemblage structure, supporting the idea that microalgae will exhibit a non-uniform response to future ocean acidification scenarios. The diversity and evenness of the assemblages decreased with CO<sub>2</sub> enrichment, whilst the dominance of a few genera increased. This is also consistent with the findings from the microphytobenthic assemblages on artificial hard substrata (Chapter 3, Johnson et al. 2012a). In addition, the observed increase in the large chain-forming genus *Striatella* with elevations in CO<sub>2</sub> is in agreement with those studies that show that ocean acidification has the ability to influence the competitive abilities amongst different size classes and different morphologies of diatoms (Tortell et al. 2008; Johnson et al. 2012a). The reduced generic diversity may also be due to increased competition for resources in high biomass biofilms at the CO<sub>2</sub> enriched stations.

Benthic meiofauna and macrofauna have the potential to influence microphytobenthic biomass, production and composition in sediments through grazing and sediment re-suspension (Reise 1992; Smith et al. 1996; Hagerthey et al. 2002; Dyson et al. 2007). There is significant variability in the pH sensitivity of different benthic fauna (Shirayama & Thornton 2005; Widdicombe & Needham 2007; Widdicombe & Spicer 2008; Barry et al. 2011), therefore increases in CO<sub>2</sub> also have the potential to alter the structure of these communities along CO<sub>2</sub> gradients. Whilst benthic faunal abundance as a potential confounding factor was not quantified in this study, it seems unlikely that it would be solely responsible for the large changes observed in the microphytobenthic assemblages. As discussed earlier, after eliminating macro invertebrate grazing as a potential co-variable, the study in Chapter 3 (Johnson et al. 2012a) demonstrated a similar positive effect of elevated CO<sub>2</sub> alone on microalgal assemblages, thereby supporting the conclusions from this study that increases in CO<sub>2</sub> may indeed stimulate the productivity of microphytobenthos in sediments (although reductions in zooplankton grazing also needs to be considered as a potential co-variable in the periphyton experiments). Hicks et al. (2011) found that infaunal grazing activity mediated complex interactions between CO<sub>2</sub> and temperature which determined the biomass of microphytobenthos. Carbon dioxide induced changes in benthic faunal assemblages may therefore have the potential to influence microphytobenthic assemblages at least to some degree. Further research is warranted to determine its relative contribution to the variability observed in these assemblages.

#### *4.4.3 Conclusion*

The microphytobenthic assemblages analysed in this study showed significant changes along a CO<sub>2</sub> gradient. Primary productivity was greatest at high CO<sub>2</sub> levels which

increased the standing crop of epilithic biofilms and microphytobenthic assemblages in the surface layers of sandy sediment. This may have important consequences for benthic trophic webs and larger scale biogeochemical processes as anthropogenic CO<sub>2</sub> emissions continue to rise. Benthic diatoms abundances increased in high  $p\text{CO}_2$ , whilst cyanobacteria in sediment appeared to require a high threshold value of  $p\text{CO}_2$  and/or elevated nutrient levels before they exhibited a positive response. The relations between some of the microalgal responses and CO<sub>2</sub> in the sediment are weaker than those observed in the biofilms, but none the less they provide further support that elevated CO<sub>2</sub> has the potential to promote algal productivity in benthic habitats. The observed alterations in the composition of biofilms under high CO<sub>2</sub> have the potential to influence the subsequent settlement of macroalgae and invertebrates, with important implications for the structure of the overlying benthic communities. Similar investigations of microphytobenthic assemblages should take place across a variety of different habitats and systems (i.e. intertidal, estuarine, tropical) to determine if the responses are comparable and to extend the spatial scale of these predictions. Whilst this study has yielded important information on the response of microphytobenthic assemblages to  $p\text{CO}_2$  enrichment, it is limited in the fact that it is merely a snapshot of a given point in time (May and September). Further investigation of these responses over different seasons would provide a more accurate indication of the expected changes in these assemblages to ocean acidification. In addition, these *in situ* observations are confined to one single CO<sub>2</sub> vent system and are therefore limited in terms of spatial replication. Repetition of this study across other CO<sub>2</sub> vent systems would strengthen predictions of microphytobenthic assemblage responses to ocean acidification over larger geographical scales. It is also important for future research to perform herbivore exclusion experiments in order to determine the relative roles of CO<sub>2</sub> enrichment and grazing pressure release as drivers of the observed responses to acidified seawater.

**Chapter 5:** Changes in endolithic microalgal and epilithic macroalgal assemblages along a CO<sub>2</sub> gradient.

Functionally important assemblages of micro and macroalgae associated with rocky substrata in shallow sub-tidal habitats were investigated along a CO<sub>2</sub> gradient off Vulcano Island, Sicily. The aims of this chapter are to characterise endolithic microalgal and epilithic algal communities along a CO<sub>2</sub> gradient, as alterations to these assemblages, due to CO<sub>2</sub>, could have important consequences for the productivity, structure, function and stability of a variety of shallow water habitats globally, particularly coral reefs. Endolithic microalgal colonisation of carbonate substrata (artificially created aragonite blocks, dead and live gastropod shells) were analysed at various points along the CO<sub>2</sub> gradient. Detailed study of the changes in some of these endolithic assemblages was not possible along the entire CO<sub>2</sub> gradient due to sample loss during winter storms. In the aragonite blocks there were no significant differences found in photosynthetic standing crop (Chl *a*) and endolithic epi-fluorescence between intermediate (S1 median  $p\text{CO}_2 = \sim 500 \mu\text{atm}$ ) and high  $p\text{CO}_2$  (S2 median  $p\text{CO}_2 = \sim 1200 \mu\text{atm}$ ). Low statistical power, however, may be limiting analysis as mean Chl *a* values at S2 were more than double those found at S1. Results indicate a positive effect of high CO<sub>2</sub> on the abundance of these endoliths, but this can only be confirmed through spatio-temporal repetition of the investigation. Analysis of shells from populations of gastropods naturally occurring along the CO<sub>2</sub> gradient revealed important differences in endolithic colonisation between alive and dead carbonate substrata. Increasing levels of CO<sub>2</sub> resulted in major shifts in the composition of epilithic algal communities; the cover of crustose coralline algae decreased, whilst turf algae increased. The ecological and biogeochemical implications of the recorded responses of endolithic and epilithic algae assemblages to elevated CO<sub>2</sub> are discussed.

## 5.1 Introduction

### 5.1.1 Endolithic microalgae

Endolithic algae, living inside rock or in the pores between mineral grains of rock, are ubiquitous in shallow hard-bottom marine ecosystems and play an important role in the degradation of calcium carbonate. Bioerosion is caused by endolithic microalgae and occurs on a wide variety of carbonate-based substrata (including calcareous shells, foraminifera, algae, live and dead coral skeletons and compact limestone / limestone sediments) worldwide (Tudhope & Risk 1985; Chazottes et al. 2002; Tribollet & Payri 2001; Tribollet et al. 2002; Carreiro-Silva et al. 2005). Microborers actively penetrate carbonate substrata, forming tunnels that conform to their body shape and are the initial colonisers of newly exposed carbonate (Kobluk & Risk 1977; Chazottes et al. 1995; Vogel et al. 2000).

Chlorophytes are major constituents of endolithic assemblages; the most common is the siphonalean chlorophyte, *Ostreobium* spp. Cyanobacteria are also widespread colonisers of endolithic habitats (Schneider 1976; Schneider & Le Campion-Alsumard 1999; Behrendt et al. 2011). Cyanobacterial bioerosion is an important process within global carbon and carbonate cycles (Schneider & Le Campion-Alsumard 1999). Initial microboring communities are dominated by the pioneer short-lived green alga *Phaeophila* spp. which is then slowly replaced (after ca. 3 months) by low light specialists such as the green alga *Ostreobium* spp. and the cyanobacteria *Plectonema terebrans* (Gektidis 1999; Vogel et al. 2000). Endolithic phototrophs play an important role in bioerosion by dissolving carbonate either via organic acid or chelating substance secretions (Le Campion-Alsumard 1979; Garcia-Pichel 2006) or by means of specialised boring organelles (Alexandersson 1975). The production of CO<sub>2</sub> during endolithic respiration may also contribute to carbonate dissolution (Garcia-Pichel 2006).



Endolithic algae have adapted to living in environments where they are exposed to low light intensities. Those living in coral reefs experience large diurnal fluctuations in pH and oxygen due to photosynthesis and respiration in the coral tissue (Shashar & Stambler 1992). Endolithic microalgal abundance can exceed more than half a million individuals per square centimetre (Schneider & Le Campion-Alsumard 1999). They form a principal food source for a variety of grazers such as molluscs, fish and echinoderms (Ogden 1977; Trudgill 1987). Positive correlations have been found between grazing and microbioerosion rates, as substratum reworking by endoliths is thought to facilitate macro-grazing (Tribollet & Golubic 2005). Phototrophic endolithic communities contribute a large proportion of net primary productivity in dead coral substrata,  $2 \text{ g C m}^{-2} \text{ day}^{-1}$ , approximately the same as epilithic biofilms (Tribollet et al. 2006b).

The destructive, weakening role of the boring activity of phototrophic endoliths has been well documented in coral reefs (Hutchings 1986; Le Campion-Alsumard et al. 1995; Glynn 1997; Garcia-Pichel 2006) and living bivalves (Raghukumar et al. 1991; Webb & Korrubel 1994; Kaehly 1999; Zardi et al. 2009). There are, however, also beneficial effects of endolithic microbes. In addition to zooxanthellae, corals harbour endolithic algae within their skeletons, most commonly the genus *Ostreobium* (Le Campion-Alsumard et al. 1995). These endoliths are thought to enhance photosynthesis in the tissues of their coral host via the translocation of fixed carbon (Ferrer & Szamt 1988; Schlichter et al. 1995, 1997). This alternative source of energy is thought to be especially important for the recovery of corals from bleaching events (Fine & Loya 2002; Fine et al. 2004; Hartman et al. 2010). In addition, endolithic algae exhibit a photoprotective role for the coral zooxanthellae during periods of high light stress (Yamazaki et al. 2008).

Numerous studies have investigated the impact of nutrients on the growth of endolithic communities, revealing a direct enhancement of microbioerosion rates with elevated nutrient levels (Pari et al. 1998, Carreiro-Silva et al. 2005, 2009). In contrast, the impact of elevated atmospheric CO<sub>2</sub> on these important communities and bioerosion rates has been largely understudied. Many studies have now demonstrated that enhanced CO<sub>2</sub> can stimulate microalgal productivity, in particular cyanobacteria (Barcelos e Ramos et al. 2007; Hutchins et al. 2007; Levitan et al. 2007; Fu et al. 2008b; Kranz et al. 2009). Therefore it may be hypothesised that there is potential for an increase in phototrophic endoliths and subsequent microbioerosion rates due to ocean acidification, especially since acidification of seawater is likely to facilitate carbonate dissolution by endolithic filaments (Tribollet et al. 2006a). In addition, Halley et al. (2005) suggested that endolithic activity may be responsible for increased carbonate dissolution in sediments under high CO<sub>2</sub> concentrations. The dissolution of calcium carbonate uses CO<sub>2</sub> which in turn buffers seawater (Kleypas et al. 1999). Increasing carbonate dissolution as a result of stimulated endolith productivity may therefore help reduce the impact of ocean acidification of seawater in coastal environments.

A short term (3 month) laboratory experiment (Tribollet et al. 2006a) found no effects of elevated CO<sub>2</sub> on endolithic metabolism, although it was hypothesised that the endoliths may have been saturated with respect to CO<sub>2</sub> under ambient conditions or limited by tank nutrient conditions. A more recent study, however, revealed that whilst elevated CO<sub>2</sub> had no effect on endolith abundance or bioeroded surface area, *Ostreobium* spp. increased its depth of penetration under *p*CO<sub>2</sub> of 750 µatm and consequently rates of carbonate dissolution were 48 % higher than under ambient CO<sub>2</sub> (Tribollet et al. 2009). This suggests that biogenic dissolution of carbonate substrata is likely to increase under ocean acidification scenarios. This has potentially serious

consequences for the integrity of coral reefs and their ability to resist erosion in an increasingly acidified ocean (Manzello et al. 2008). However, it also raises the possibility of a potential ocean acidification feedback mechanism in which increased rates of carbonate dissolution may buffer the effects of coastal acidification. Considering the significance of endolithic bioerosion in the global carbonate cycle, there is clear need for further research and as yet there have been no *in situ* studies addressing the impacts of long term exposure to elevated CO<sub>2</sub> on endolithic communities in natural marine systems. This chapter presents the findings of an investigation into endolithic communities along a volcanic CO<sub>2</sub> gradient off the island of Vulcano, NE Sicily. The main aim was to investigate the endolithic assemblages in experimental CaCO<sub>3</sub> substrata (aragonite blocks and limpet shells) installed at different points along the CO<sub>2</sub> gradient. Standing photosynthetic crop (Chl *a* content) and community composition of the phototrophic endoliths that colonised these substrata over a period of ca. 7 months were determined in order to investigate the *in situ* responses of these communities to CO<sub>2</sub> enrichment.

#### 5.1.2 Epilithic algal communities

Epilithic algal communities are diverse assemblages of calcifying crusts (crustose coralline algae) and algal turfs growing upon rock. They form a crucial component of shallow water ecosystem trophodynamics by contributing to a large proportion of the net primary productivity (Klumpp & McKinnon 1989), providing a significant source of carbon to herbivorous grazers (Klump & Polunin 1990). Due to intensive grazing (Hatcher 1983) the epilithic algal community is maintained at a small standing crop but has high turnover rates and fast rates of primary productivity (Rogers & Salesky 1981; Carpenter 1985).

Calcifying crusts are abundant and widespread on sub-tidal rocky coasts worldwide (Steneck 1986). They are a significant component of the understory of perennial canopies of algae in temperate waters (Irving et al. 2004) whilst in the tropics they are important for maintaining coral dominated reef systems as they cement and stabilise the coral reef matrix (Bjork et al. 1995) and can induce the settlement of coral larvae (Heyward & Negri 1999). Algal turfs are ubiquitous multi-specific and inconspicuous associations of unicellular and short (< 10 mm in height) simple filamentous algae together with juvenile stages of macroalgae. Whilst turfs are also integral to the healthy functioning of shallow water habitats (Klump & McKinnon 1989; Knowlton 2001; Diaz-Pulido & McCook 2004) their expansion can lead to ecological problems. Many turf species are ephemeral and require increased resource availability to be competitively superior to perennial species (Airoldi et al. 2008). Therefore, on coasts subjected to anthropogenic chemical and biological alterations, opportunistic turfs can replace perennial canopies of macroalgae such as kelp forests (Connell et al. 2008) and can contribute to coral-algal phase shifts (McManus & Polsenberg 2004). Fast growing turfs can rapidly colonise bare substrata and overgrow crusts (Russell & Connell 2005).

Ocean acidification has the potential to cause profound structural and functional changes in epilithic algal communities, as elevated CO<sub>2</sub> is likely to reduce the calcification of crusts (Martin & Gattuso 2009) whilst enhancing primary productivity in turfs (Connell & Russell 2010). Furthermore, short term mesocosm experimentation has shown that elevated CO<sub>2</sub> can act synergistically with increased temperature, nutrients and light to facilitate the expansion of filamentous turfs at the expense of calcifying crusts (Russell et al. 2009, 2011a; Connell & Russell 2010). In this chapter an investigation into the composition of epilithic algal communities along a CO<sub>2</sub> gradient took place in order to assess the impacts of *in situ* long term CO<sub>2</sub> exposure on these functionally important benthic components.

## 5.2 Methodology

### 5.2.1 Endolithic Microalgae

The endolithic microalgae investigation was conducted between 5<sup>th</sup> October 2011 and 2<sup>nd</sup> May 2012 at stations R4 and S1-S3 along the CO<sub>2</sub> gradient off the island of Vulcano, NE Sicily. R4 was selected over the remaining reference stations R1-R3 as the seabed at this site was more suitable for placement of the experimental units (flat sandy surface as opposed to large boulders).

See Chapter 2 for full site and station description, and methods used to assess carbonate chemistry.

#### 5.2.1.1 Preparation of the CaCO<sub>3</sub> experimental substrata

Endolithic assemblages along the CO<sub>2</sub> gradient were investigated through the installation of both natural and artificially created CaCO<sub>3</sub> substrata. Aragonite blocks representing coral skeletons were created artificially to produce standard experimental units. The colonisation of naturally sourced CaCO<sub>3</sub> substrata (dead limpet shells, *Patella vulgata* and dead coral, *Acropora* spp.) was also investigated so that the effects of high CO<sub>2</sub> on endoliths in a range of materials could be determined. These three types of experimental substrata were intended to be representative of coral rubble and newly dead CaCO<sub>3</sub> surfaces in coastal environments. The weight and size of each substrata was measured before installation to determine loss of CaCO<sub>3</sub> to erosion, and the naturally sourced substrata were scrubbed clean to remove epilithic algae.

To create  $\text{CaCO}_3$  blocks, aragonite sand (Caribsea Aragamax, sugar-sized grains; 0.1-1 mm), White Portland (Snowcrete) cement (which contains calcium carbonate and is free of any pigments and additives), and granular salt crystals were mixed together in the ratio 2:1:1. To this, freshwater was added to make a thick slurry mixture. The mixture was placed in polystyrene moulds (25.5 cm x 19.5 cm x 2.5 cm) and plastic tubes were inserted to make holes for attachment. The mixture was allowed to set for 24-36 hours before removing the moulds and plastic tubes. The blocks were then immersed in freshwater to allow for curing over a one month period with daily fresh water changes. During this time the salt crystals dissolved creating a porous structure more representative of natural coral and allowing faster endolith colonisation. These blocks were then cut into smaller experimental units (8.5 x 6.5 x 5.5 cm) by a professional stone cutter (see Appendix VI for photographs).

#### *5.2.1.2 Installation of experimental $\text{CaCO}_3$ substrata*

At each station (R4, S1-S3) the  $\text{CaCO}_3$  blocks ( $n = 10$ -20 per station), limpet shells ( $n = 10$  per station) and coral pieces (various lengths; 3-13 cm,  $n = 4$  per station) were weighed, measured and attached to cement bases (divided between 2 bases per station; 'base A' comprised of blocks and coral, 'base B' comprised of blocks and limpets). The experimental substrata were attached to the cement bases using cable ties and marine epoxy putty (Z-Spar Splash Zone). On the 5th October 2010 the cement bases were placed on the seabed at a depth of ~1-2 m at each station. At S2 an additional cement base (C) containing just  $\text{CaCO}_3$  blocks ( $n = 10$ ) was placed in an area of reduced light, and therefore in conditions expected to reduce endolith infestation (Zardi et al. 2009), in order to examine the relationship between endolithic abundance and carbonate dissolution. Due to the logistical problems of creating the necessary procedural controls

for low light (i.e. algal overgrowth of cages) and due to potential confounding cage effects, a naturally shaded area was selected for this particular set of  $\text{CaCO}_3$  blocks (pier pilings). Using a light probe meter (Extech 403125) light reduction under the pier was calculated at ca. 56 %. The experimental bases were removed ca. 7 months later on the 27th April 2011.

#### *5.2.1.3 Investigating in situ populations of gastropods for endoliths*

The endolithic assemblages found in shells naturally occurring along the  $\text{CO}_2$  gradient were also investigated. Populations of two species of inter-tidal gastropods (the limpet *Patella caerulea* and the sea snail *Osilinus turbinata*) were sampled at stations R1-R3 and S1-S3.

A total of 20 live specimens of each species were collected from the inter-tidal zone at each station on the 2<sup>nd</sup> May 2012.

#### *5.2.1.4 Processing of the $\text{CaCO}_3$ substrata*

Following removal of macro epilithic fauna and flora, the experimental  $\text{CaCO}_3$  substrata were re-weighed and re-measured. The aragonite blocks however, could not be re-weighed as it was not possible to measure their dry weight due to the porosity of the blocks increasing the water content (and drying the blocks would have resulted in chlorophyll degradation), therefore the amount of carbonate dissolution had to be determined through changes in cubic volume only. All  $\text{CaCO}_3$  substrata, naturally occurring and transplanted, were scrapped of epilithic algae using a tooth brush and scalpel. This revealed a thin yellow-green zone of endoliths associated with the

carbonate skeleton below. All samples were then immediately frozen at -20 °C and stored at -80 °C (< 3 weeks) upon return to the laboratory.

#### 5.2.1.4.1 Chlorophyll extraction

For the aragonite blocks, Chl *a* was extracted from 1-2 cm<sup>2</sup> chippings taken from the upper surface using a hammer and chisel. Photographs were then taken to calculate the precise surface area using the image analysis software, Image J. The surface area of the limpet shells was determined using the formula for calculating the curved surface area of a cone ( $CSA = \pi \times r \times s$ , where  $r$  = radius,  $s$  = slant height). The surface area of the species *O. turbinata* proved more difficult due to its coiled shell, therefore shells were weighed instead. All substrata were ground to a fine powder using a pestle and mortar-like manual crushing device. Chlorophyll was extracted from the powdered CaCO<sub>3</sub> substrates using 100% hot ethanol and the absorbance of each sample at 632, 649, 665, 969 and 750 nm was measured on a spectrophotometer (Cecil CE2011). Three replicate readings were taken at each absorbance to calculate an average value of chlorophyll *a* for each sample. The total amount of Chl *a* was calculated using the quadrichroic equation of Richie (2008) and expressed per unit surface area ( $\mu\text{g cm}^{-2}$ ) for the blocks and limpets and per unit dry weight for the snails ( $\mu\text{g g}^{-1}$ ).

#### 5.2.1.4.2 Epi-fluorescence analysis

A confocal laser scanning microscope (CLSM) was used to determine the abundance of chlorophytes and cyanobacteria within the thin upper surface layer of the carbonate substrates (aragonite blocks, *P. vulgata* and *P. caerulea*). The samples were viewed using a BioRad 2000 (Bio-Rad, UK), excitation 488 nm; emission 570-590/70 and 660-



700 nm. A total of 30 images were taken (x 10 mag) at random locations across the surface of the substrates (limpet shells were broken into pieces using a hammer) and the percentage cover of chlorophyte and cyanobacteria fluorescence was digitally quantified per image using the Image J software. An average percentage cover was then calculated for each sample from the 30 images.

### 5.2.2 *Epilithic algal communities*

Epilithic algal communities were sampled in April 2012 at stations R1-R3 and S1-S3.

The EAC were studied by taking photographs of the upper surface of haphazardly selected, medium sized rocky boulders ( $\sim 10\text{-}20\text{ cm}^3$ ) removed from the shallow sub-tidal zone ( $< 0.5\text{ m}$ ). The percentage surface area covered by the two components of epilithic algal communities; turfs ( $< 10\text{ mm}$  high) and crustose coralline algae, in a  $10\text{ cm}^2$  area per boulder was estimated using Image J analysis.

### 5.2.3 *Statistical Analysis*

Differences in  $\text{CaCO}_3$  substrata, endolithic assemblages and epilithic algal communities between stations were tested using one-way ANOVA, and multiple pairwise comparison post hoc tests (Tukey's Test) were performed where differences were significant. Data that failed tests for normality (Shapiro-Wilk) and homogeneity (Levene Median test) were transformed (arc sin and ln). When transformations were unsuccessful, data was analysed by Kruskal-Wallis one way analysis of variance on ranks and multiple pairwise comparison post hoc tests (Dunn's method). A Pearson's correlation test was used to test for a significant relationship between endolithic

abundance and erosion of  $\text{CaCO}_3$ . These statistical analyses were performed using SigmaPlot 11.0.

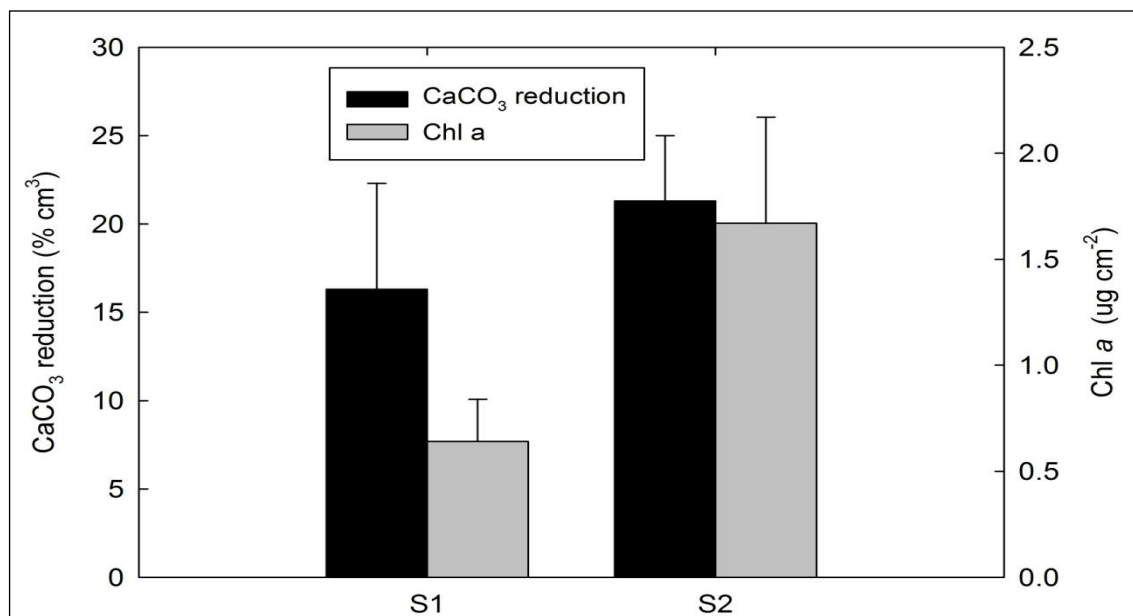
### 5.3 Results

#### *5.3.1 Endolithic microalgae assemblages in experimentally installed aragonite blocks*

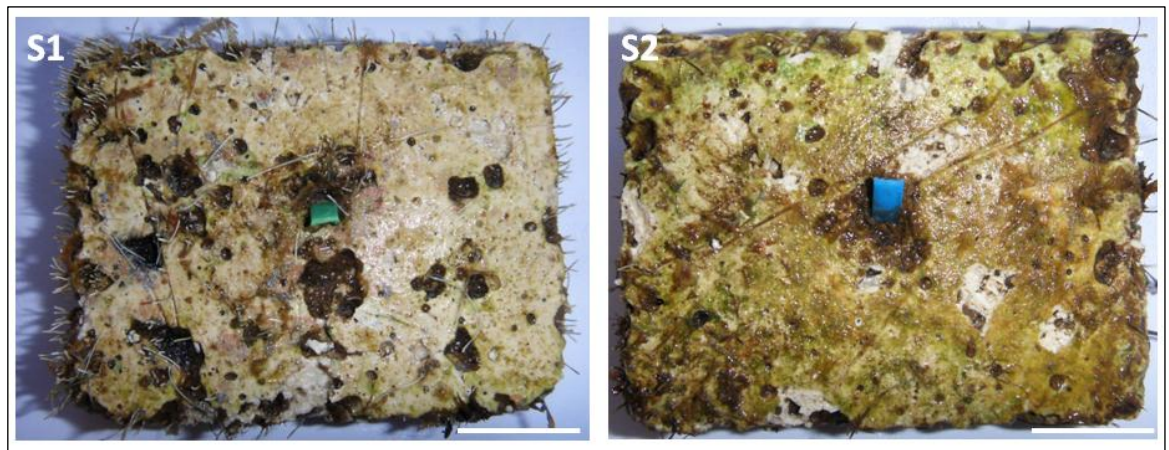
Following the ca. 7 month installation period, experimental bases survived at only two stations (S1 and S2), presumably due to severe winter storms. Crucially, this resulted in the loss of reference samples at station R4. Furthermore, at stations where the experimental bases had remained, one base from each station had become dislodged from its original placement and was found upturned on the sea bed. This will have resulted in a significant reduction in light levels for an unknown length of time, thereby rendering the substrates attached to these bases unsuitable for comparison with those that remain in their original position. Despite an incomplete sample set with a limited number of replicates, analysis of the remaining substrata from intermediate and a high  $p\text{CO}_2$  (S1 median = 510  $\mu\text{atm}$  , S2 median =1244  $\mu\text{tm}$ ) may still provide useful information on potential responses and trends of endolithic assemblages to increasing  $\text{CO}_2$ .

A summary of the various data derived from the transplanted  $\text{CaCO}_3$  substrata is presented in Appendix IV and photographs showing them after their colonisation after ca.7 months are shown in Appendix VI. Due to the varying conditions of light the experimental bases were exposed to, only substrata on bases S1A and S2B can be directly compared. Unfortunately, only one of these bases contained limpets, therefore no comparative data on limpets can be derived, and only two coral skeletons survived again on non comparable bases so these were omitted from the analysis. The loss of

CaCO<sub>3</sub> (expressed as a reduction in cubic volume) from the aragonite blocks was greater in the lowest pH (S2; mean % reduction cm<sup>3</sup> = 21.3 ± 3.7, Fig. 5.1) than in the intermediate (S1: mean % reduction cm<sup>3</sup> = 16.3 ± 6.0, Fig. 5.1). There was however no significant difference detected between the two stations (ANOVA;  $F_{(1,6)} = 0.004$ ,  $P = 0.951$ ), reflecting the low number of replicates (S1;  $n = 3$ , S2;  $n = 5$ ). Similarly, the mean Chl *a* concentration in aragonite blocks at S2 B ( $1.67 \pm 0.5 \mu\text{g cm}^{-2}$ ) was between 2-3 fold greater of that extracted from blocks in S1A ( $0.64 \pm 0.2 \mu\text{g cm}^{-2}$ ) indicating a larger abundance of endolithic algae (Fig. 5.1 and also highlighted in Fig. 5.2), however no differences could be detected statistically (ANOVA;  $F_{(1,6)} = 2.539$ ,  $P = 0.162$ ). The mean epi-fluorescence of endolithic chlorophytes was also greater in blocks at S2 compared to S1 ( $29.4 \% \pm 3.2$ ,  $19.9 \% \pm 3.5$ , respectively) however this difference could not be verified statistically (ANOVA;  $F_{(1,4)} = 4.005$ ,  $P = 0.116$ ). Cyanobacteria epi-fluorescence remained relatively low in both stations ( $< 2 \%$ ) and no significant differences could be detected (ANOVA;  $F_{(1,4)} = 3.797$   $P = 0.123$ ).



**Fig. 5.1** Mean reduction in CaCO<sub>3</sub> and mean Chl *a* concentrations in the aragonite blocks at stations S1 ( $n = 3$ ) and S2 ( $n = 5$ ) following the ca. 7 month endolithic colonisation period.

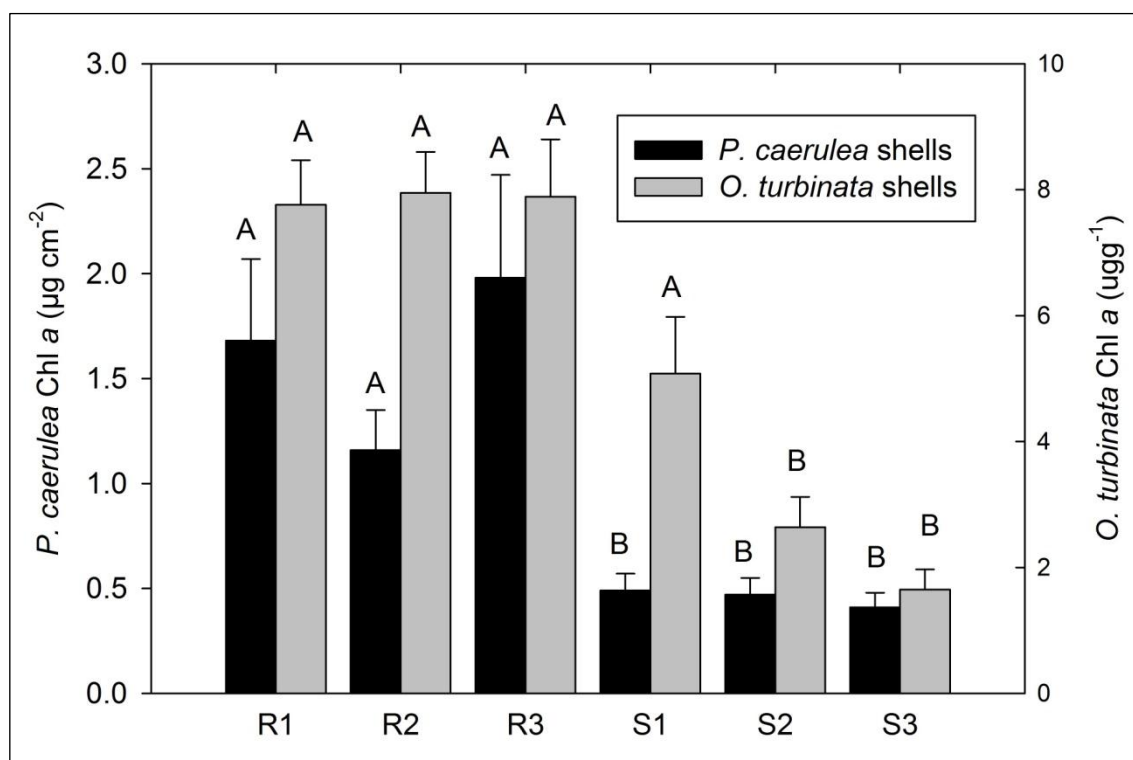


**Fig. 5.2** Photographs of aragonite blocks following ca. 7 months of installation (Oct 2011- April 2012) at stations S1 and S2 on the Vulcano CO<sub>2</sub> gradient. Removal of overgrowing macroalgae revealed green patches of endolithic chlorophyte colonisation in the upper surface layer of the aragonite blocks are visible in both images, but this appears to be more extensive in S2, scale bar = 2 cm.

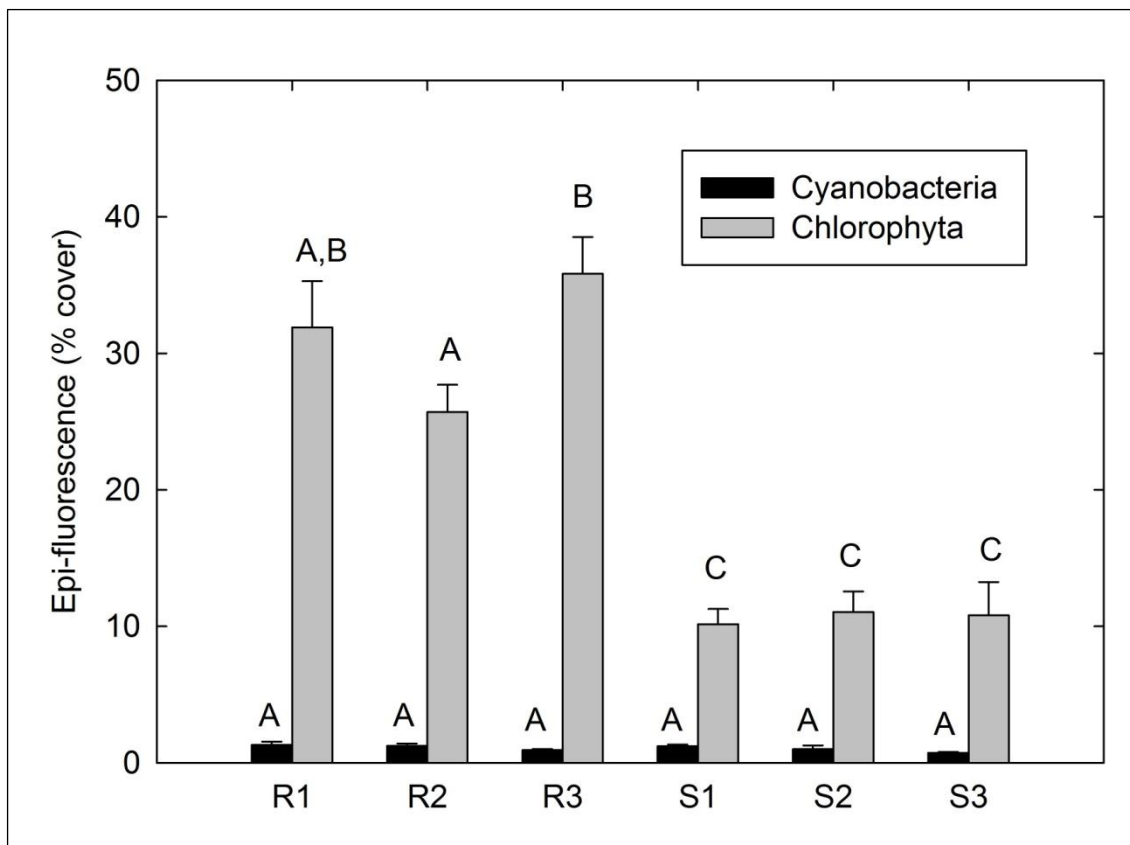
There was a higher mean Chl *a* concentration in aragonite blocks placed under reduced light conditions (base C) at S2 compared with those in ambient light (base B),  $2.73 \text{ ugcm}^{-2} \pm 0.5$  and  $1.67 \pm 0.3 \text{ ugcm}^{-2}$  respectively. However no significant difference could be detected statistically between these two bases (ANOVA;  $F_{(1,12)} = 1.930$ ,  $P = 0.190$ ), again potentially due to low replication (B;  $n = 5$ , C;  $n = 11$ ). There was also no difference in the reduction of CaCO<sub>3</sub> between blocks at bases b and c (ANOVA:  $F_{(1,14)} = 0.006$ ,  $P = 0.938$ ). Furthermore, no significant relationship could be detected between the abundance of endolithic algae (concentration of Chl *a*) and the erosion of CaCO<sub>3</sub> (% reduction in cubic volume) in substrates from bases S2 B & C (Pearson's correlation;  $R = -0.0237$ ,  $P = 0.923$ ,  $N = 19$ ).

### 5.3.2 Endolithic microalgae assemblages in natural populations of gastropods

In the inter-tidal gastropod shells there was a significant reduction in the amount of Chl *a* as CO<sub>2</sub> levels increased (Fig. 5.3). Chlorophyll *a* was two–four fold greater in the reference stations compared with the CO<sub>2</sub> enriched stations. Along the CO<sub>2</sub> gradient, there were significant differences recorded in the Chl *a* concentration of *P.caerulea* (ANOVA;  $F_{(5,112)}=11.759$ ,  $P < 0.001$ ) and *O.turbinata* (ANOVA;  $F_{(5,111)} = 24.302$ ,  $P < 0.001$ ). In *P. caerulea* shells no significant differences could be detected between the three reference stations nor between the three stations exposed to high CO<sub>2</sub> (Tukey,  $P > 0.05$ ). In *O. turbinata* shells Chl *a* was significantly lower in stations S2 and S3 than the reference stations and S1 (Tukey,  $P < 0.05$ ), no significant differences could be detected between the latter four stations (Tukey,  $P > 0.05$ ). The epi-fluorescence findings from the *P. caerulea* shells support the pattern observed in the Chl *a* data; there was a significant reduction in chlorophyte epi-fluorescence between the reference stations and the CO<sub>2</sub> enriched stations (Fig. 5.4 & images in Appendix V; ANOVA;  $F_{(5,42)} = 25.330$ ,  $P < 0.001$ ). There was no significant difference recorded in cyanobacteria epi-fluorescence (ANOVA;  $F_{(5,42)} = 1.548$ ,  $P = 0.196$ ),



**Fig. 5.3** Mean (+ SE) Chl *a* concentration in two species of inter-tidal gastropods, *Patella caerulea* (µg cm<sup>-2</sup>) and *Osilinus turbinata* (µg g<sup>-1</sup>) along the Vulcano CO<sub>2</sub> gradient in May 2012 ( $n = 20$  per species, per station). Means with the same letters are not significantly different ( $P > 0.05$ ).



**Fig. 5.4** Mean (+ SE) epi-fluorescence of cyanobacteria and chlorophyta in the shells of *Patella caerulea* along the Vulcano CO<sub>2</sub> gradient in May 2012 ( $n = 8$ , per station).

There were differences in endolithic colonisation between the naturally occurring live limpets and the empty shells installed on the experimental bases at station S2 (Fig. 5.5). Significant differences in the amount of Chl *a* (ANOVA;  $F_{(1,26)} = 19.772$ ,  $P < 0.001$ ) and chlorophyte epi-fluorescence (ANOVA;  $F_{(1,14)} = 86.125$ ,  $P < 0.001$ ) were detected between dead and live limpet shells. Dead shells contained almost three times the concentration of Chl *a* than the live shells (station S2: dead shells; mean =  $1.38 \pm 0.2 \mu\text{g cm}^{-2}$ , live shells; mean =  $0.47 \pm 0.08 \mu\text{g cm}^{-2}$ ). Similarly the epi-fluorescence of chlorophytes was over three-fold greater in the dead shells compared with the naturally occurring limpets (mean % cover =  $37.9 \pm 2.5$  and  $11.0 \pm 1.5$ , respectively).

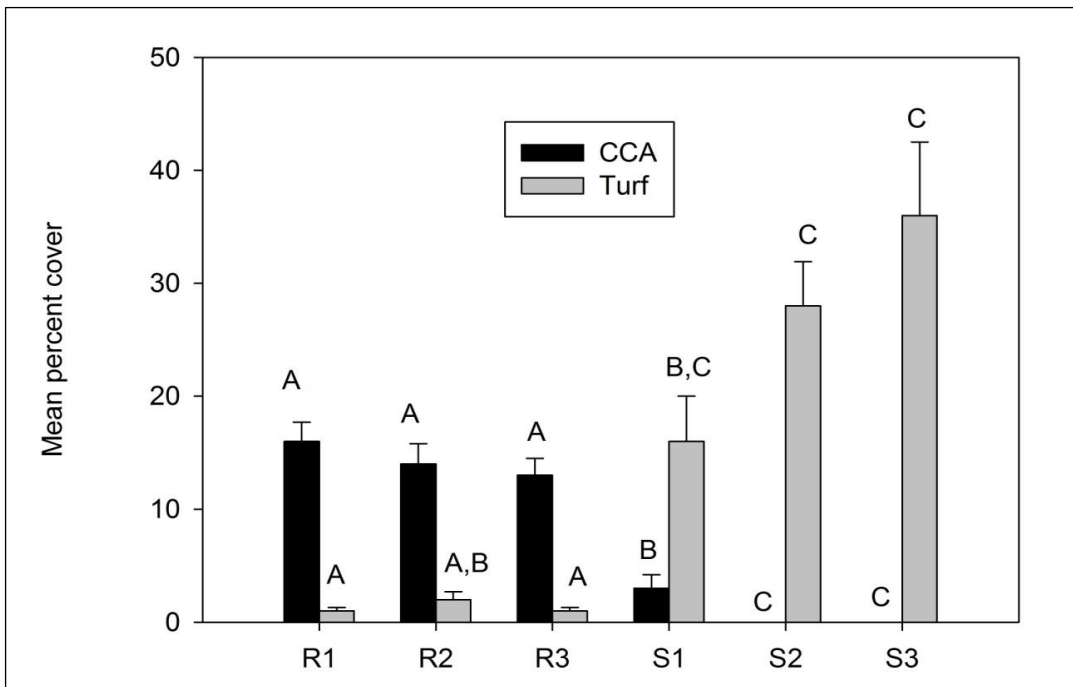


**Fig. 5.5** Photographs highlighting the differences in the extent of endolithic colonisation between live and dead limpets at station S2. The left image shows live *P. caerulea* whilst the right image shows extensive endolith colonisation (green/yellow zones) in dead *P. vulgata* shells that were installed at S2 for 7 months. Scale bars = 1 cm.

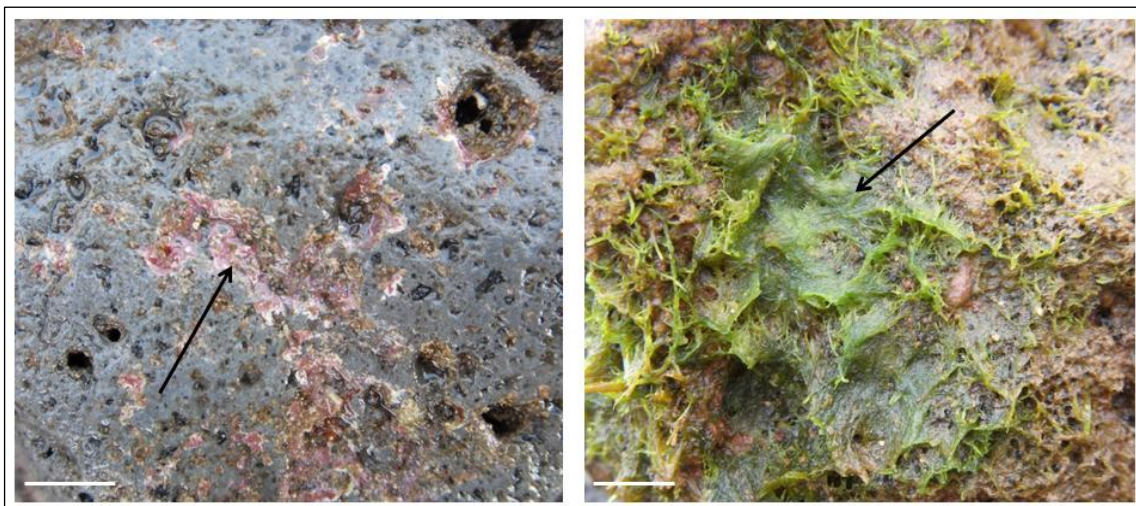
### 5.3.3 Epilithic algal communities

Major shifts in the composition of epilithic algal communities were recorded along the CO<sub>2</sub> gradient (Fig. 5.6 & 5.7). The abundance of crustose coralline algae decreased as CO<sub>2</sub> increased. At the reference stations mean percentage cover was between 13-16 % whilst at S1 it was reduced to 3 % and was absent at station S2 and S3. There were significant differences in the percentage cover of crustose coralline algae along the CO<sub>2</sub> gradient (Kruskal-Wallis;  $H_5 = 85.241$ ,  $P < 0.001$ ). Turfs, on the other hand, showed a significant increase with increasing CO<sub>2</sub>; the percentage cover remained relatively low at all three reference stations (1-2 %) but increased concomitantly with rising levels of CO<sub>2</sub>, cover reached a maximum at S3 (36 %). There were significant differences in the cover of turf algae along the CO<sub>2</sub> gradient (Kruskal-Wallis;  $H_5 = 73.992$ ,  $P < 0.001$ ). These turfs consisted primarily of filamentous Chlorophytes c.f *Pseudochlorodesmis furcellata* (Fig. 5.7).





**Fig. 5.6** Changes in the composition of epilithic algal communities: crustose coralline algae (CCA) and turfs; short (< 10 mm in height) simple filamentous algae, along the Vulcano CO<sub>2</sub> gradient in April 2012 ( $n = 20$  per station). Means with the same letters are not significantly different ( $P > 0.05$ ).



**Fig. 5.7** Photographs of epilithic algal communities on boulders removed from the shallow sub tidal zone at R2 (left image) and S3 (right image) highlighting the shifts in composition from crustose coralline algae to turf domination along the Vulcano CO<sub>2</sub> gradient, scale bar = 1 cm.

## 5.4 Discussion

### 5.4.1 Endolithic assemblages

Aragonite blocks installed along a CO<sub>2</sub> gradient were used to simulate newly dead coral surfaces in order to investigate the changes in assemblages of colonising endolithic microalgae. Unfortunately the loss of samples from two stations, R4 in particular, makes it difficult to draw firm conclusions from this sample set, and a small number of replicates from the stations where the cement bases did survive the winter storms limits statistical power. Despite this, however, some interesting trends appear to be emerging which warrant discussion and highlight the importance of repeating the investigation. Visual inspection of the aragonite blocks, Chl *a* concentration and epi-fluorescence imaging analysis indicated a higher abundance of endolithic chlorophytes at station S2 compared with S1, although this could not be confirmed statistically, perhaps due to small sample sizes. Carbon acquisition mechanisms in endolithic algae are poorly understood and research into the responses of endolithic microalgae to CO<sub>2</sub> is limited to a couple of short term experiments, however there does appear to be some support for the observations made in this study. The microboring filaments of the dominant endolith *O. quekettii* have been shown to grow faster and deeper under elevated CO<sub>2</sub> than at ambient levels (Tribollet et al. 2009). Furthermore it has been suggested that endolithic microalgae may not have CCM and therefore depend on passive diffusion of CO<sub>2</sub> (Tribollet et al. 2006a, 2009) which may explain the positive trend of endolithic chlorophyte abundance with elevated CO<sub>2</sub> observed here.

Carbonate dissolution by microboring endolithic algae is a major process in the carbon cycle, particular in coral reef systems (Tribollet & Golubic et al. 2005; Tribollet et al.

2009) so it is important to investigate the responses of these algae to rising CO<sub>2</sub> and the resulting consequences for biogenic dissolution. Relative to blocks from S1, the mean reduction of carbonate (expressed in cubic volume) in the blocks from S2 was greater although differences could not be confirmed statistically. It is not however possible to differentiate between the relative contribution of chemical and biogenic carbonate dissolution therefore the potential effects of greater endolith abundance on carbonate dissolution cannot be determined in this case. However, when pH is removed as a co-variable no significant relationship could be detected between the abundance of endolithic microalgae and the amount of carbonate dissolution, again possibly hindered by low statistical power. This was investigated by installing substrates within the same area of low pH exposure but under different light intensities (ambient vs 54 % reduction, bases B and C at S2 respectively) in order to manipulate the amount of endolithic growth for comparison of biogenic dissolution. Surprisingly, instead of the expected endolith reduction, the blocks in reduced light exhibited higher mean Chl *a* concentration than those in ambient light. This may be due to the extensive cover of macroalgae on blocks in ambient light, reducing light penetration to the surface of the blocks relative to those in the shaded environment or the effects of photoinhibition under higher light intensities.

Despite the fact that no significant differences were found between endolithic microalgae in the aragonite blocks the observed trends suggest that there may be a potential positive effect of high CO<sub>2</sub> on these assemblages which may be confirmed through repeated investigations with larger and complete sample sets. If biogenic carbonate dissolution is found to increase with elevated CO<sub>2</sub> *in situ*, as predicted from previous laboratory experiments (Tribollet et al. 2009) this has potentially profound consequences for biogeochemical cycles and models, particularly concerning ocean acidification feedback mechanisms. However, stimulated endolithic algae growth may

also have serious consequences for coral reefs. Their ability to accumulate and grow will be compromised under higher rates of biogenic dissolution, particularly as acidified seawater is likely to facilitate carbonate dissolution by endolithic filaments (Tribollet et al. 2006a) and as lowered aragonite saturation states may impair the precipitation of inorganic reef cements (Manzello et al. 2008). Further research is clearly required particularly concerning the potential benefits of increased endolith abundance and growth on the translocation of fixed carbon to the coral host, as this may partially offset the negative effects of biogenic dissolution. Furthermore as this investigation was conducted in a temperate ecosystem, future studies should aim to utilise CO<sub>2</sub> gradients in tropical vent systems to account for any potential differences between geographical regions.

In order to investigate endolithic assemblages across the entire length of the CO<sub>2</sub> gradient, the shells of live gastropods were sampled, thereby allowing the comparison between CO<sub>2</sub> enriched stations and reference stations as this was not possible through the aragonite block experiment. The analysis of these carbonate substrata revealed surprising results, contradicting the findings obtained from the aragonite blocks and from previous experiments (Tribollet et al. 2006a, 2009). Both Chl *a* and epi-fluorescence analysis revealed a significant reduction of endolithic microalgae under elevated CO<sub>2</sub>. This is unlikely to be the result of any potential negative effects of reduced pH on endolithic microalgae as no such responses have been observed in the previous experiments (Tribollet et al. 2006a, 2009). One potential explanation may be due to changes in grazing pressure along the CO<sub>2</sub> gradient. The teeth of chitons and limpets are well suited for rasping on hard surfaces and are often found grazing on hard substrata where they excavate the surface to remove epi- and endolithic algae (Steneck & Waitling 1982b). Grazing upon epilithic and endolithic algae constantly removes the surface of substrata thereby providing more light which in turn stimulates the

penetration of microboring endolithic filaments (Schneider & Torunski 1983). External surfaces of limpet shells can often be eroded by smaller grazing limpets (Eekout et al. 1992), limpet grazing can be particularly powerful due to the possession of hard radular teeth (Branch 1981). The abundance of several calcified macro invertebrate grazers including limpets has been found to significantly decrease with high CO<sub>2</sub> along a natural gradient (Hall-Spencer et al. 2008) and was also observed along this particular gradient (pers. obs). Therefore the potential reduction in calcified macro invertebrate grazing on the gastropod shells at the CO<sub>2</sub> enriched stations may account for the lower endolithic abundance relative to the reference stations.

It may be argued that low pH at the CO<sub>2</sub> enriched stations, like grazing, may also be expected to contribute to removal of the surface of carbonate shells due to chemical erosion. This, however, seems unlikely as live calcified animals, including *P. caerulea*, have the ability to up-regulate calcification when exposed to low pH to counteract the effects of dissolution, often being able to calcify and grow at even faster than normal rates (Findlay et al. 2009; Rodolfo-Metalpa et al. 2011). This is particularly relevant when it comes to understanding the differences in the extent of endolithic colonisation between live and dead limpet shells observed in this study. Chlorophyll *a* and chlorophyte epi-fluorescence were significantly greater in transplanted dead *P. vulgata* shells at station S2 compared with naturally occurring live *P. caerulea*. These differences seem to be too large for limpet species-specific differences in endolithic colonisation. Instead they may relate to the fact that in live shells there is a great degree of biological control on dissolution (Findlay et al. 2009). Therefore, as shell growth rates have been shown to be higher in *P. caerulea* at CO<sub>2</sub> vent sites (Rodolfo-Metalpa et al. 2011) relative to ambient conditions, this may confine the penetration of endoliths to the uppermost surfaces of the shell only. Dead shells, on the other hand, dissolve faster (Rodolfo-Metalpa et al. 2011) and therefore more light becomes available within shells,

stimulating the penetration of endoliths to deeper depths and possibly sustaining populations at higher abundances. It should be noted however, that due to the mobility of live populations of gastropods, unlike transplanted shells, the length of time individual animals spend at each station along the gradient over the 7 month period cannot be accurately determined, therefore variations in exposure to high CO<sub>2</sub> may be a potential factor influencing the endolith growth.

In summary, based on the limited dataset collected here, elevated CO<sub>2</sub> had no significant effect on endolith assemblages in aragonite substrata. There was however a trend of greater mean abundances between intermediate (~500 µatm) and high CO<sub>2</sub> (~1200 µatm), indicating that elevations in CO<sub>2</sub> may have the potential to stimulate endolithic chlorophytes in coral reefs (assuming the findings from this temperate system readily translate to tropical systems). This can only be confirmed through repeating the experiment in order to increase sample sizes and compare differences between CO<sub>2</sub> enriched stations and reference stations. Endolithic assemblages in live gastropod shells decreased with increasing CO<sub>2</sub>, highlighting important differences between dead and live carbonate substrata as environments for endolithic microalgae. As a result endolithic assemblages from the carbonate substrata of live fauna are not an accurate representation of those found occurring within dead CaCO<sub>3</sub> substrata. Furthermore endolithic microboring activity has been found to be much greater in dead substrata (Tribollet & Payri 2001) so these should form the focus of future investigations. Endolithic cyanobacteria were recorded at low abundances and showed no significant changes in response to CO<sub>2</sub> in both aragonite substrata and live limpet shells. These findings are comparable to other studies on benthic cyanobacteria (Johnson et al. 2012a; Tribollet et al. 2006b, and Chapter 4).

#### 5.4.2 *Epilithic algal communities*

Elevations in CO<sub>2</sub> caused significant alterations to the composition of sub-tidal epilithic algal communities with dominance shifting from crustose coralline algae to turf algae. These changes are analogous to the shifts recorded in macroalgal communities in other CO<sub>2</sub> vent studies (Hall-Spencer et al. 2008; Fabricius et al. 2011; Porzio et al. 2011) which also observed the replacement of calcified macroalgae with fleshy species. The absence of crustose coralline algae from these communities at elevated CO<sub>2</sub> contributes to a growing body of laboratory (Kuffner et al. 2008; Martin & Gattuso 2009; Büdenbader et al. 2011) and field based (Martin et al. 2008; Fabricius et al. 2011) evidence which indicate that this group of algae are highly vulnerable to the effects of ocean acidification. The reduction in crustose coralline algae is more likely due to the direct effect of seawater acidification as opposed to an inverse competitive relationship between crustose coralline algae and turf cover as has been previously demonstrated in other studies of calcified vs non calcified macro algae (Kuffner et al. 2008; Hofman et al. in review). This is due to the fact that turf algae cover, despite expanding with increases in CO<sub>2</sub>, rarely fully occupied the available space on boulders sampled (max. mean cover = 36 %). The loss of the functionally important crustose coralline algae component of the epilithic algal communities has profoundly serious consequences for the integrity and resilience of variety of benthic habitats worldwide. Coral reefs, in particular, are likely to be severely impacted by a reduction in crustose coralline algae due to its associated negative impacts on coral larvae recruitment (Doropoulos et al. 2012) and reef stability, particularly if bioerosion rates are also set to increase with ocean acidification (Manzello et al. 2008; Tribollet et al. 2009).

Within the CO<sub>2</sub> enriched stations, green turfs were found to occupy ~ 15-30 % more boulder space than under ambient conditions. The concomitant increase in turf

abundance with CO<sub>2</sub> levels indicates that these algae are perhaps benefiting physiologically from increased rates of photosynthetic carbon fixation and growth as reported in other species of fleshy macroalgae (Gao et al. 1991, 1993b; Kübler et al. 1999). The responses observed in this study are supported by a recent investigation of rocky shore community responses (Graziano et al. in review). This study also documented the negative effects of high CO<sub>2</sub> on calcified algae and the elevated CO<sub>2</sub>-induced proliferation of turfs along two different Mediterranean CO<sub>2</sub> gradients. (including the one off Vulcano Island). In addition, previous experiments have recorded increases in turf algae recruitment, percentage cover, dry mass and/or photosynthetic efficiency (Russell et al. 2009; Connell and Russell 2010; Russell et al. 2011a) under elevated CO<sub>2</sub>. The results of a few other studies, however conflict with the current findings. In a short-term mesocosm experiment turf algae showed no response to elevations in CO<sub>2</sub> (Doropoulos et al. 2012) and, surprisingly, an *in situ* survey at a different vent site in the Mediterranean recorded a reduction in the cover of turfs along a gradient of increasing CO<sub>2</sub> (Porzio et al. 2011). The reason for the discrepancies between the two vent systems remains unclear although it may be due to site-specific differences in nutrient levels and/or herbivore pressures. Further research is required along different CO<sub>2</sub> gradients worldwide in order to elucidate these variations in responses observed between different field studies.

#### 5.4.3 Conclusion

These findings indicate that increasing CO<sub>2</sub> levels may perturb benthic macro and micro algal communities in rocky shore ecosystems. Changes in the productivity and composition of these photoautotrophic communities may have cascade effects for a variety of shallow water ecosystems globally, particularly coral reefs. Coral reef habitats have been predicted to rapidly decline over the next few decades as increasing



CO<sub>2</sub> will lead to global warming-induced mass bleaching events and the onset of ocean acidification will retard coral growth (Veron et al. 2009). The findings from this study highlight further negative consequences of increasing CO<sub>2</sub> on coral reefs. The potential for CO<sub>2</sub> induced expansion of endolithic microalgae within coral skeletons may increase rates of biogenic dissolution which will further impede the ability of corals to grow and accumulate. Increasing CO<sub>2</sub> may also indirectly impact coral reef habitats through perturbations of epilithic algal communities. The loss of crustose coralline algae under high CO<sub>2</sub> will also contribute to further reef erosion, whilst an expansion of turfs favours a shift towards a macroalgae dominated system. These indirect effects of elevated CO<sub>2</sub> should therefore also be integrated into predictions concerning the fate of these ecological and economically valuable habitats.

**Chapter 6:** The ecological and physiological responses of *Padina pavonica* (Phaeophyta, Dictyotaceae) to elevated CO<sub>2</sub>

This chapter presents an assessment of the ecology and physiology of the calcified phaeophyte *Padina pavonica* along natural  $p\text{CO}_2$  gradients. Whilst calcified coralline algae (Rhodophyta) appear to be especially vulnerable to ocean acidification, there is a lack of information concerning calcified brown algae (Phaeophyta), which are major producers of calcium carbonate and organic matter in shallow coastal waters.

Populations growing along a CO<sub>2</sub> gradient off Vulcano Island, Sicily, were investigated to determine the relative changes over spatial scales, indicating important information on the effects of elevated CO<sub>2</sub> on the ecology and physiology of this common macroalga. The CaCO<sub>3</sub> content of *P. pavonica* significantly decreased with CO<sub>2</sub> enrichment along the gradient, however, in contrast to studies of other calcified macroalgae, the benthic cover of this species increased. Significant increases in the photosynthetic rates of *P. pavonica* and reductions in the abundance of grazing sea urchins at high CO<sub>2</sub> may explain the success of decalcified *P. pavonica* in the low pH environment studies. These findings indicate that *P. pavonica* may be one of the ecological winners of ocean acidification.

Data from this chapter have now been published in the journal Global Change Biology:

**Johnson VR**, Russell BD, Fabricius KF, Brownlee C, Hall-Spencer JM (2012b). Temperate and tropical brown macroalgae thrive, despite decalcification, along natural CO<sub>2</sub> gradients. Global Change Biology 18: 2792-2803

## 6.1 Introduction

Calcified macroalgae are widely distributed in marine habitats. They play a crucial role in the ecology of coastal systems and deposition of their calcium carbonate is an important aspect of the global carbon cycle (Nelson 2009). Marine macroalgal calcifiers are represented by > 100 genera, spanning all three of the major algal divisions (Hillis-Colinvaux 1980), expressing different  $\text{CaCO}_3$  mineralogy and calcification mechanisms. At present, however, we lack an understanding of how these diverse characteristics are likely to affect macroalgal sensitivity to ocean acidification. *Padina* is a common genus in warm, shallow seas and is one of only two genera of phaeophyta that calcifies, the other is *Newhousia*, described by Kraft et al. (2004). *Padina pavonica* (Linnaeus) Thivy grows abundantly on Mediterranean shores. It is a seasonal annual macroalga (Airoldi 2000) present as fan-shaped thalli during spring and summer, and in some areas through to autumn (Einav et al. 1995; Piazzzi et al. 2002). On the surface of *P. pavonica* thalli, calcium carbonate is deposited as aragonite needles, forming concentric bands of white precipitate (Borowitzka et al. 1974; Okazaki et al. 1986). *Padina* is an important producer of both calcium carbonate and organic matter in shallow waters in the tropics and subtropics (Bathurst 1971; Milliman 1974). Carbonate production rates of *Padina* have been calculated to be around  $240 \text{ gm}^{-2}\text{yr}^{-1}$ , considerably higher than the other main tropical erect calcified algal genera *Halimeda* ( $50 \text{ gm}^{-2}\text{yr}^{-1}$ ) and *Pencillus* ( $30 \text{ gm}^{-2}\text{yr}^{-1}$ ) (Wefer 1980).

Present knowledge of the effects of ocean acidification on calcified macroalgae is mostly derived from studies investigating the impacts of elevated  $\text{CO}_2$  on calcifiers with high magnesium calcite skeletons, such as the family Corallinaceae (Anthony et al. 2008; Kuffner et al. 2008; Martin et al. 2008; Martin & Gattuso 2009; Semesi et al. 2009; Gao & Zheng 2010; Büdenbender et al. 2011). The surface seawater saturation

state of aragonite ( $\Omega$  3-4) is greater than that of high magnesium calcite ( $\Omega$  2-3), so algae that precipitate the latter are expected to have greater difficulty producing their  $\text{CaCO}_3$  skeletons under increasing  $\text{CO}_2$  than aragonite species (Kleypas et al. 1999). As a consequence, aragonitic species have been relatively overlooked in ocean acidification studies. Furthermore, the responses of calcified phaeophyta are virtually unknown (Porzio et al. 2011). Since they are not obligate calcifiers (they deposit  $\text{CaCO}_3$  extracellularly), their response may differ from that of Corallinaceae which are obligate calcifiers with intercellular deposition (within cell walls).

Several roles of macroalgal calcification have been proposed. It has been thought to offer structural defence, providing mechanical resistance to herbivores (Littler & Littler 1980, 1983; Steneck 1982a), minimise the consequences of herbivore damage (Padilla 1993), increase the ability for bicarbonate and nutrient assimilation through the generation of protons (McConnaughey & Whelan 1997), improve the photosynthetic performance of the alga (McConnaughey 1998) and protect the alga from excess irradiance (Bürger & Schagerl 2010). Changes in calcification have the potential to impact the physiological and ecological fitness of calcified macroalgae through reductions in photosynthetic efficiency, thallus rigidity, growth rates and competitive abilities (Nelson 2009). There have been numerous studies that have revealed the negative impacts of reduced seawater carbonate saturation states on the settlement, calcification rates, growth and abundance of calcified macroalgae (Hall-Spencer et al. 2008; Jokiel et al. 2008; Kuffner et al. 2008; Martin et al. 2008; Andersson et al. 2009; Martin & Gattuso 2009; Robbins et al. 2009; Russell et al. 2009; Price et al. 2011; Sinutok et al. 2011; Büdenbender et al. 2011). Increasing concentrations of  $\text{CO}_2$  can, on the other hand, enhance productivity and growth in both non-calcified (Gao et al. 1991, 1993b; Kübler et al. 1999; Connell & Russell 2010) and calcified macroalgae (Reiskind et al. 1988; Gao et al. 1993a; Semesi et al. 2009).

Most ocean acidification studies on calcified macroalgae to date have been single species laboratory experiments lasting a year at most (Martin & Gattuso 2009). Such experiments provide important information on species' responses to acute increases in CO<sub>2</sub> but fail to account for the effects of chronic and long term exposure. They are also unrepresentative of natural ecosystems since, for example, they remove the effects of species interactions (Barry et al. 2011). In contrast to short laboratory experiments, CO<sub>2</sub> gradients in natural settings, where whole ecosystems have been exposed to elevated levels of *p*CO<sub>2</sub>, allow us to investigate changes in the interactions, competition, predation and/or herbivory that involve long-lived metazoan species in benthic marine ecosystems.

Ocean acidification has the potential to reduce top-down biological control of benthic biodiversity (Widdicombe & Spicer 2008). Sea urchins are dominant grazers in many marine habitats and play an important role in controlling the structure and composition of macroalgal communities. They often act as keystone species (Sala et al. 1998) and, as a consequence, reduction in their abundance or removal from an ecosystem can result in rapid colonisation of benthic habitats by macroalgae (Villouta et al. 2001; Behrens & Lafferty 2004; Hernández et al. 2008). Sea urchins are particularly susceptible to reductions in pH (Miles et al. 2007) and a mean pH of 7.8 appears to be the critical level below which Mediterranean sea urchins do not survive (Hall-Spencer et al. 2008).

Adverse impacts of ocean acidification on echinoderms would be likely to have significant consequences at the ecosystem level (Barry et al. 2010; Dupont et al. 2010). It has the potential to release algae from the control of grazing by sea urchins, resulting in cascade effects throughout benthic food webs, with potentially profound implications for the structure and function of marine communities.

Investigations of macroalgal communities living near CO<sub>2</sub> vents have started to reveal important information on the expected ecological changes that may occur with increasing CO<sub>2</sub> (Hall-Spencer et al. 2008; Martin et al. 2008; Fabricius et al. 2011; Porzio et al. 2011). An increase in the cover of fleshy macroalgae has been recorded alongside a reduction in the cover of calcified species in both temperate (Hall-Spencer et al. 2008; Porzio et al. 2011) and tropical benthic systems (Fabricius et al. 2011). This chapter presents the findings of an investigation into *P. pavonica* along the CO<sub>2</sub> gradient off the island of Vulcano. This is the first *in situ* assessment of the long term impacts of low pH and high CO<sub>2</sub> on *P. pavonica* calcification and photosynthetic performance. It also investigates *Padina*–sea urchin population dynamics along CO<sub>2</sub> gradients as interactions between macroalgae and grazers can drive ecological changes in benthic habitats on temperate (Sala 1998; Hernández et al. 2008) and tropical shores (McClanahan 1994; Mumby et al. 2006).

## **6.2 Material and Methods**

### *6.2.1 Study sites and carbonate chemistry*

This study was conducted at stations R1-R3 and S1-S3 along a CO<sub>2</sub> gradient off the island of Vulcano, NE Sicily between September 2010 and September 2011.

See Chapter 2 for a detailed description of site and sampling stations, and methods for assessing the carbonate chemistry.

### 6.2.2 *Padina* and sea urchin abundance

To take into account the temporal variability in macroalgae populations, surveys were conducted on three separate occasions; autumn (September) 2010, spring (May) 2011 and autumn 2011. For this part of the study, an additional four sampling stations were selected along the gradient; one located between S1 and S2 (at mean pH 7.97, IQ = 7.79-8.15,  $n = 16$ ), three at ~20 m intervals between S1 and the end of the gradient (at mean pH 8.08, IQ: 8.02-8.11; pH 8.16 IQ: 8.10-8.21; pH 8.20, IQ: 8.19-8.23,  $n = 6-22$ ) to allow *P. pavonica* and sea urchin abundance surveys to occur along the full length of the CO<sub>2</sub> gradient. The heterogenic physical nature of the benthos in the shallow subtidal habitat required the use of a stratified sampling protocol to assess to abundance of *P. pavonica*. Twenty quadrats (50 cm x 50 cm) were haphazardly placed (“blind throws”) within a 15 x 3 m (depth < 0.5 m) survey area (pre-defined based on its suitability for *P. pavonica* growth) at each station. Within each quadrat the percentage cover of *P. pavonica* was estimated and in the September 2010 and May 2011 surveys the total number of sea urchins (*Paracentrotus lividus* and *Arbacia lixula*) was also recorded.

### 6.2.3 Calcium carbonate content determination and crystal examination

In September 2010, mature *P. pavonica* fronds ( $n = 30$ ) were collected from each of the six sampling station (R1-R3, S1-S3) and stored in 70 % ethanol until analysis. To determine the calcium carbonate (CaCO<sub>3</sub>) content of each frond the weight loss after acidification method was conducted (Martone 2010). Fronds were dried, weighed and decalcified in hydrochloric acid (1N) overnight. This resulted in the complete dissolution of a thin layer of CaCO<sub>3</sub>; the fronds were then re-dried and re-weighed. The

CaCO<sub>3</sub> content (expressed as a percentage of dry weight) was calculated from the difference between dried mass and decalcified dry mass.

Aragonite crystals on the frond surface were examined for size and abundance using a scanning electron microscope (SEM). Fronds from each station ( $n = 3$ ) were fixed in glutaraldehyde for 1-2 hours, and then stored in 1x PBS buffer (phosphate buffered saline) until examination. As the size and number of crystals has been reported to vary with age of frond segment (Hills-Colinvaux 1980) only the apical segments of *Padina* fronds were compared between stations. Prior to viewing under the SEM (JEOL JSM 5600 LV) samples were air dried, mounted on aluminium stubs with carbon adhesive tape and coated in gold. For each of the 18 samples, 5 images were taken at random locations over the frond surface (calcified regions only, see Fig. 6.4a) and the average length and width of 10 randomly selected crystals per image was measured digitally using Image J software (v 1.43, National Institutes of Health, Bethesda, MD, USA), and the number of crystals within 5 randomly selected 5  $\mu\text{m}$  x 5  $\mu\text{m}$  area were counted and averaged ( $n = 15$  per station).

#### 6.3.4 Photosynthesis

Photosynthetic capacity and performance of *P. pavonica* was investigated through measurements of photosynthetic pigment (Chl *a* and  $c_1+c_2$ ) concentrations and Chl *a* fluorescence, respectively. These physiological measurements were performed in summer months (May & Sept) when algal productivity is high. For pigment analysis, fronds were collected in September 2010 and September 2011 from each sampling site ( $n = 40$  per station), rinsed in distilled water and frozen for transportation back to the laboratory. Fronds were collected between 8am-10am to avoid the confounding effect of



light intensity, in particularly noon photoinhibition, on chlorophyll content (Hanelt et al. 1993; Hädar et al. 1996). To prevent chlorophyll degradation during storage samples were kept at -20 °C during the sampling period on Vulcano and at -80 °C when longer periods occurred between analysis. Chlorophyll was extracted from all samples within < 2 weeks of sampling.

Prior to extraction fronds (~ 0.70 g samples) were homogenized in 90 % acetone by pestle and mortar. Chlorophyll was then extracted in 90 % acetone at 4 °C for 24 hours. The absorbance of each sample at 630, 664 and 750 nm (background absorbance), was measured (3 replicate readings were taken from each sample to obtain an average) using a Cecil CE2011 spectrophotometer, and the concentration of chlorophyll *a* and *c* ( $c_1 + c_2$ ) in the sample was calculated using the equations of Ritchie (2006). The volume of the solvent (in weight / g) and the weight of the frond were then used to provide a final calculated reading of chlorophyll ( $\mu\text{g mg}^{-2} \text{fw}$ ), values from the two September sampling periods were pooled and a mean was calculated for each station.

In May 2011 the effective quantum yield (*Y*), and relative electron transport rates (*rETR*) of freshly collected, light-adapted fronds ( $n = 6$  per station, stored in seawater from site of collection), were measured in small dishes using a Diving PAM fluorometer (Walz-Germany).

$$Y = F'_m - F_t / F'_m$$

$$rETR = Y \times PAR \times 0.5$$

where;  $F'_m$  = maximum fluorescence yield of light adapted fronds,  $F_t$  = steady-state level of fluorescence under illumination at time *t* (Genty, 1989), PAR = photosynthetic active radiation and 0.5 is a constant assuming both PSI and PSII absorb equal amounts of the incoming photons (Beer et al. 1998; Hanelt et al. 2003).

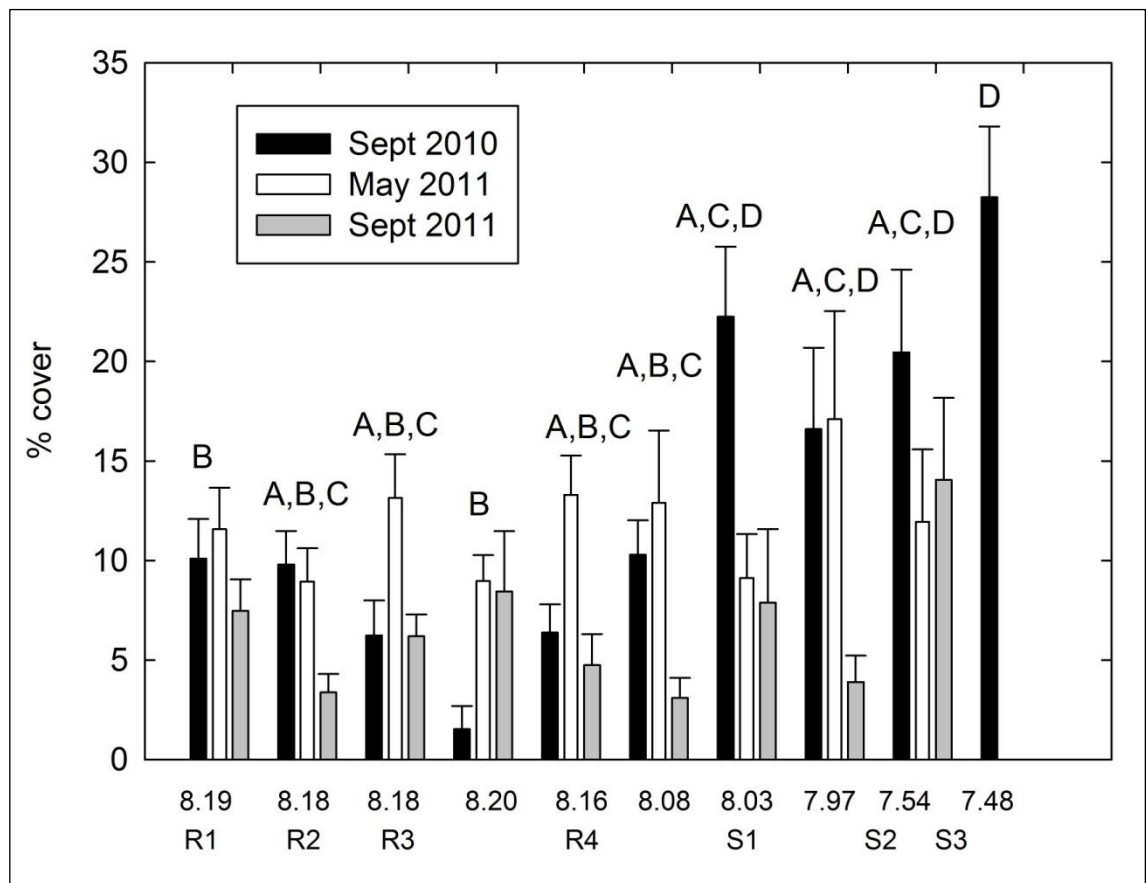
The absorption of light may differ between algal types, requiring determination of the species specific absorption factor for direct comparisons of ETR, however the effects of CO<sub>2</sub> on the photosynthetic activity are only being measured on one species, therefore *r*ETR can be used. Rapid light curves (RLC) were applied to assess the light saturation behaviour of fronds across each of the six sampling stations. RLC data can be useful for assessing photosynthetic capacity and potential over a wide range of ambient light intensities (Ralph & Gademann 2005). The Diving-PAM was set to deliver red pulse-modulated light at 655 nm followed by steps of actinic light from 1 to 3344  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  delivered every 20 s over a period of 160 s (other settings were as follows: gain = 4, actinic light factor = 0.5, light curve intensity = 5, saturation width = 0.8, saturation intensity = 3, signal damping = 2).

#### 6.2.5 Statistical Analysis

The variations in *Padina* populations among stations were analysed using one-way ANOVA, and pairwise multiple comparison procedures (Tukey's test) were used where differences were significant. Data was transformed (log and arcsin) when necessary to meet the assumptions for normality and homogeneity. Data that failed tests for normality (Shapiro-Wilk) and homogeneity (Levene Median test) after transformations were analysed by Kruskal-Wallis one way analysis of variance on ranks and Dunn's post hoc pairwise comparison tests. All statistical analyses were performed using SigmaPlot 11.0.

### 6.3 Results

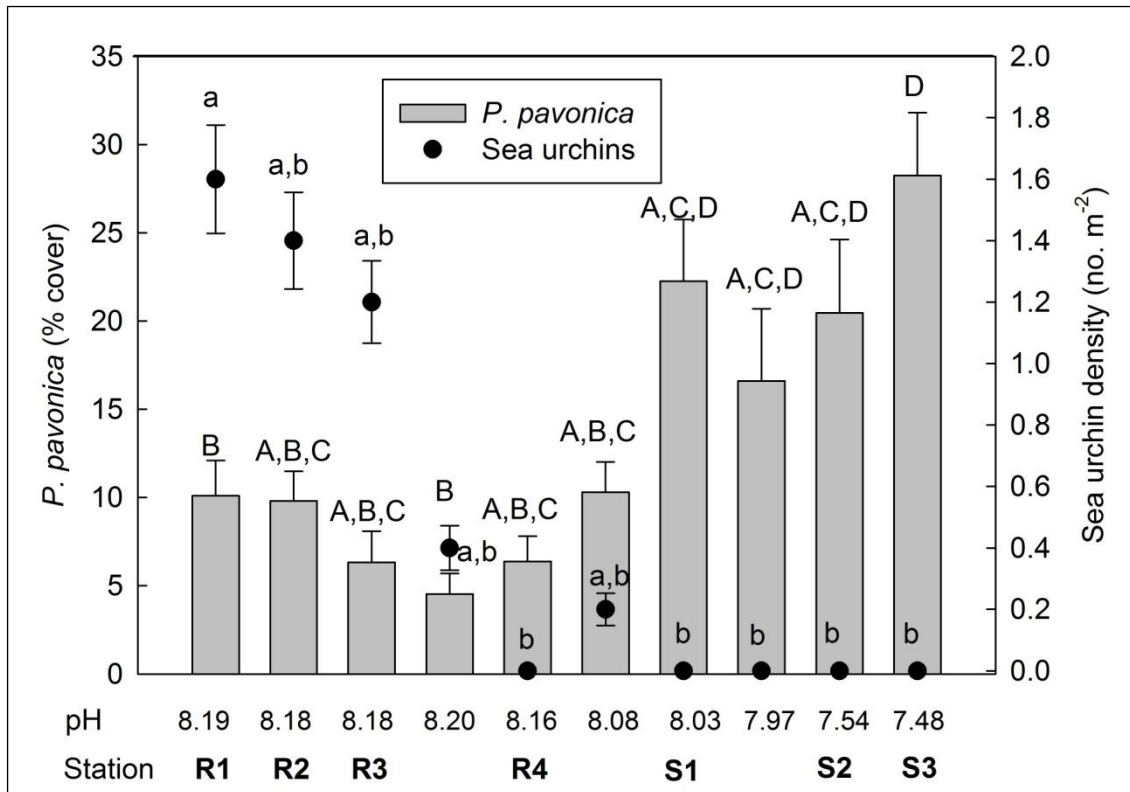
In general, there was an increasing trend in *P. pavonica* abundance along the gradient of increasing  $p\text{CO}_2$  (Fig. 6.1). In the September 2010 sampling period there were significant differences found in mean percentage cover of *P. pavonica* along the Vulcano  $\text{CO}_2$  gradient (Fig. 6.1; ANOVA:  $F_{(9,190)} = 6.651$ ,  $P < 0.001$ ). The benthic cover of *P. pavonica* increased with increasing proximity to the  $\text{CO}_2$  vents and was significantly greater at S3 ( $28 \% \pm 3.55$ , Tukey,  $P < 0.05$ ) than at all those stations with mean  $\text{pH} > 8.08$ . No differences in cover could be detected between the  $\text{CO}_2$  enriched stations (S1-S3). There was no significant difference in *P. pavonica* cover between stations along the  $\text{CO}_2$  gradient in May 2011 (ANOVA  $F_{(8,171)} = 0.851$ ,  $P = 0.590$ ) and September 2011 (ANOVA:  $F_{(8,171)} = 1.029$ ,  $P = 0.416$ ) although, crucially, S3 could not be assessed during these sampling periods due to anthropogenic benthic disturbances (shallow sub-tidal dredging and small scale marina activities) which may have also had some impact on the *Padina* populations surveyed at S2.. There were variations in the response of *P. pavonica* along the  $\text{CO}_2$  gradient between the three different sampling periods. However, a two-way ANOVA revealed there was no significant individual effect of season ( $F_{(2,532)} = 1.068$ ,  $P = 0.345$ ) and station ( $F_{(9,532)} = 0.948$ ,  $P = 0.482$ ) on *P. pavonica* cover, and there was no significant interaction between these two factors ( $F_{(16,532)} = 1.067$ ,  $P = 0.384$ ).



**Fig. 6.1** Mean percentage cover (+SE) of *P. pavonica* ( $n = 20$  per station) along the Vulcano CO<sub>2</sub> gradient. Sampling occurred between Sept 2010-Sept 2011 at ~ 20 m intervals along the gradient and at the four reference stations (R1-R4). No significant difference was found between stations in May 2011 and September 2011. September 2010 means with the same letters are not significantly different ( $P > 0.05$ ).

In contrast to *P. pavonica*, sea urchins decreased with increasing CO<sub>2</sub> and were absent at stations approaching the vents (S1-S3). Significant differences in sea urchin densities were found along the gradient in September 2010 (Fig. 6.2; Kruskal Wallis:  $H_9 = 101.328$ ,  $P < 0.001$ ) and in . May 2011 (Kruskall-Wallis:  $H_9 = 101.328$ ,  $P < 0.001$ ). In May the highest densities were found in the reference stations (R1 =  $5 \text{ urchins/m}^2 \pm 0.359$ , R2 =  $7.2 \pm 0.381$ , R3 =  $6.2 \pm 0.359$ ) but were recorded absent at S1-S3. However, no significant correlation could be detected between sea urchin density and *P.*

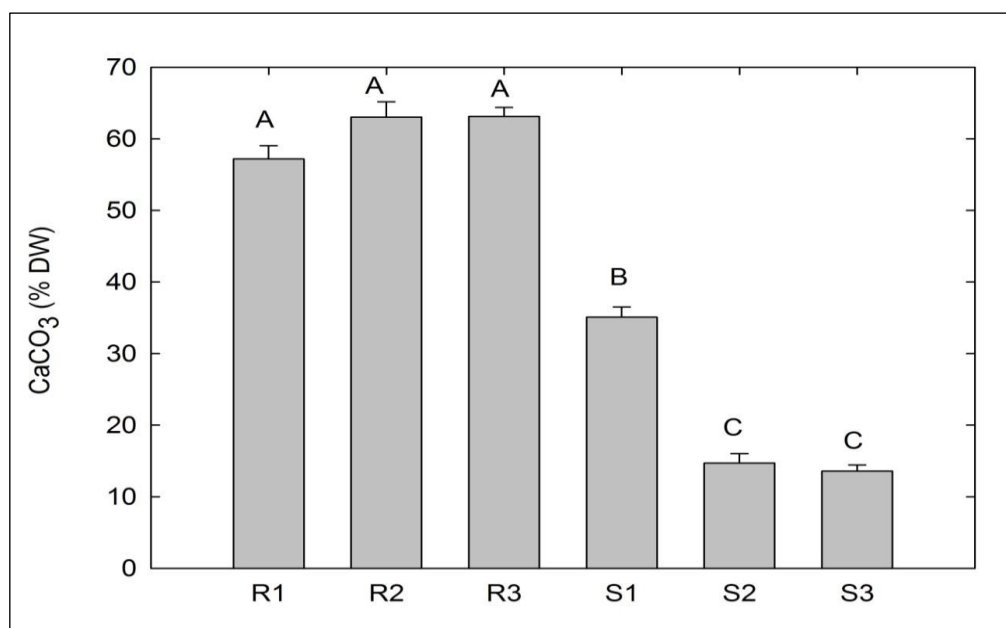
*pavonica* abundance in both September 2010 (Pearson's correlation;  $R = -0.533$ ,  $P = 0.112$ ,  $N = 10$ ) and May 2011 (Pearson's correlation;  $R = -0.274$ ,  $P = 0.475$ ,  $N = 9$ ).



**Fig. 6.2** Changes in the mean density ( $\pm$  SE) of sea urchins alongside changes in *P. pavonica* mean benthic cover ( $\pm$  SE) ( $n = 20$  quadrats per station) along the Vulcano  $\text{CO}_2/\text{pH}$  gradient in September 2010. Means with the same letters (*Padina* = upper case, sea urchins = lower case) are not significantly different ( $P > 0.05$ ).

The  $\text{CaCO}_3$  content in *P. pavonica* fronds decreased in the  $\text{CO}_2$  enriched stations relative to the reference stations. There was significantly different between all six sampling stations along the gradient (Fig 6.3 & 6.4a; Kruskal Wallis test:  $H_5 = 145.773$ ,  $P < 0.001$ ). When compared to fronds at the reference stations (R1-R3)  $\text{CaCO}_3$  content was significantly lower in S1 ( $35.1 \% \pm 1.424$ ), S2 ( $14.7 \% \pm 1.311$ ) and S3 ( $13.6 \% \pm 0.869$ ) (Dunn,  $P < 0.05$ ). Table 6.1 and Figure 6.4b presents the results of morphometric analysis of the aragonite crystals on the surface of *P. pavonica* fronds. As pH decreased the abundance of crystals increased and became thinner. There was a

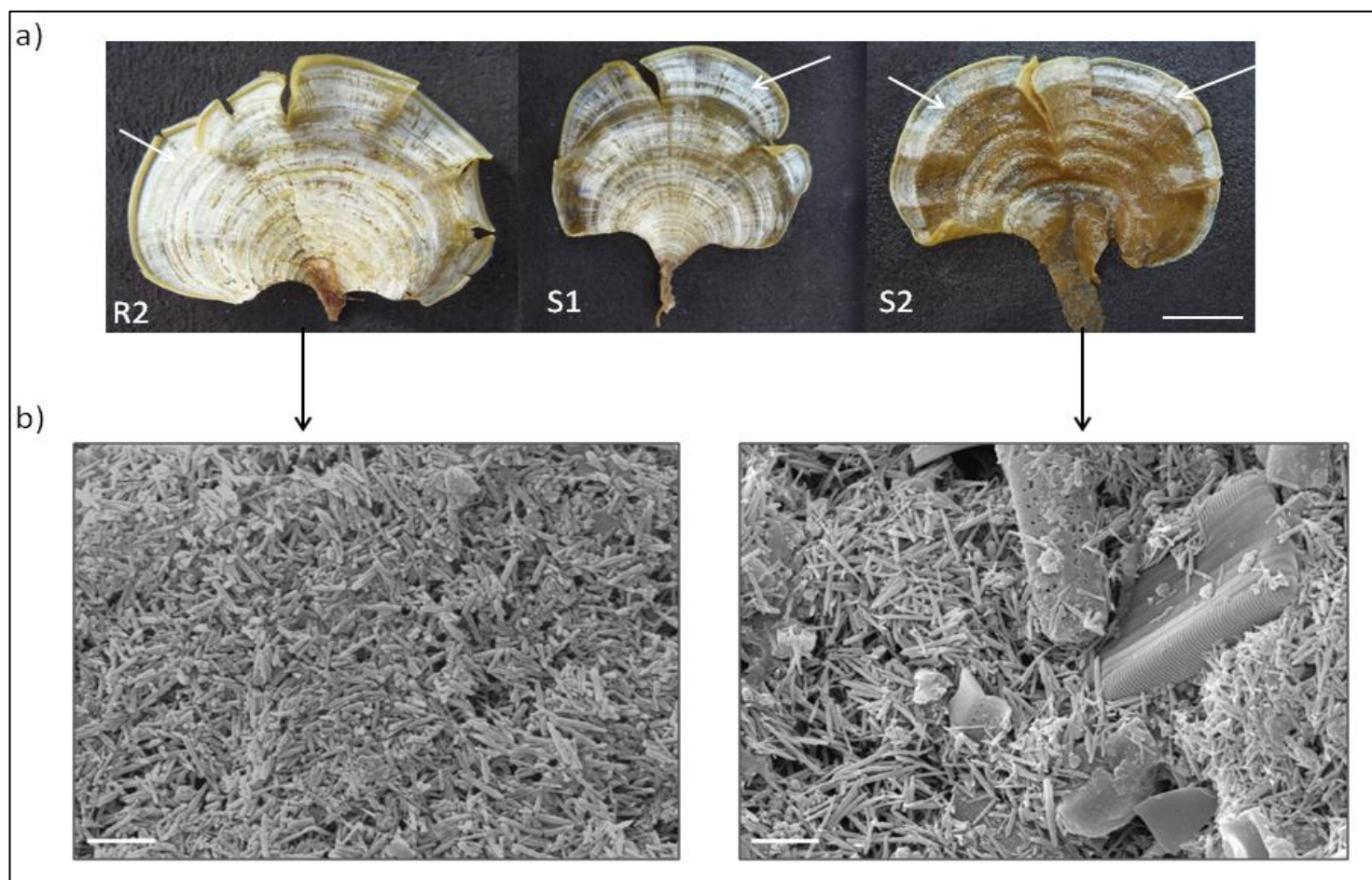
significant difference in mean aragonite crystal abundance on the surface of *P. pavonica* fronds between stations (ANOVA:  $F_{(5,84)} = 8.430$ ,  $P < 0.001$ ). S3 had a significantly greater abundance than S2, S1, R1, R2 and R3 (Tukey,  $P < 0.05$ ). Morphometric analysis of the aragonite crystals showed that their lengths were also significantly different (ANOVA:  $F_{(5,84)} = 5.662$ ,  $P < 0.001$ ) The crystals were significantly longer in S1 (Tukey,  $P < 0.05$ ) but no differences could be detected between the remaining stations (Tukey,  $P > 0.05$ ). There was a significant difference in crystal width between stations along the gradient (ANOVA:  $F_{(5,84)} = 3.881$ ,  $P < 0.001$ ). R3 had significantly wider crystals ( $0.21 \mu\text{m} \pm 0.01$ ) than S3 ( $0.17 \mu\text{m} \pm 0.01$ ) (Tukey,  $P < 0.05$ ), however, whilst there was an overall trend of decrease along the gradient, no other differences could be detected between the remaining stations (Tukey,  $P > 0.05$ ).



**Fig. 6.3** Mean (+ SE) CaCO<sub>3</sub> content of *P. pavonica* fronds along the Vulcano CO<sub>2</sub> gradient in September 2010 ( $n = 30$  per station). Means with the same letters are not significantly different ( $P > 0.05$ ).

**Table 6.1** Mean ( $\pm$  SE) abundance, length and width of aragonite crystals deposited by *P. pavonica* along the CO<sub>2</sub> gradient. Data derived from SEM analysis of fronds ( $n = 3$  fronds per station), over calcified apical regions only (see frond images in Fig 6.4a), therefore do not reflect total means for whole fronds, \* indicates sig value of one way ANOVA tests between stations ( $P < 0.05$ ). Means with the same letters are not significantly different ( $P > 0.05$ ).

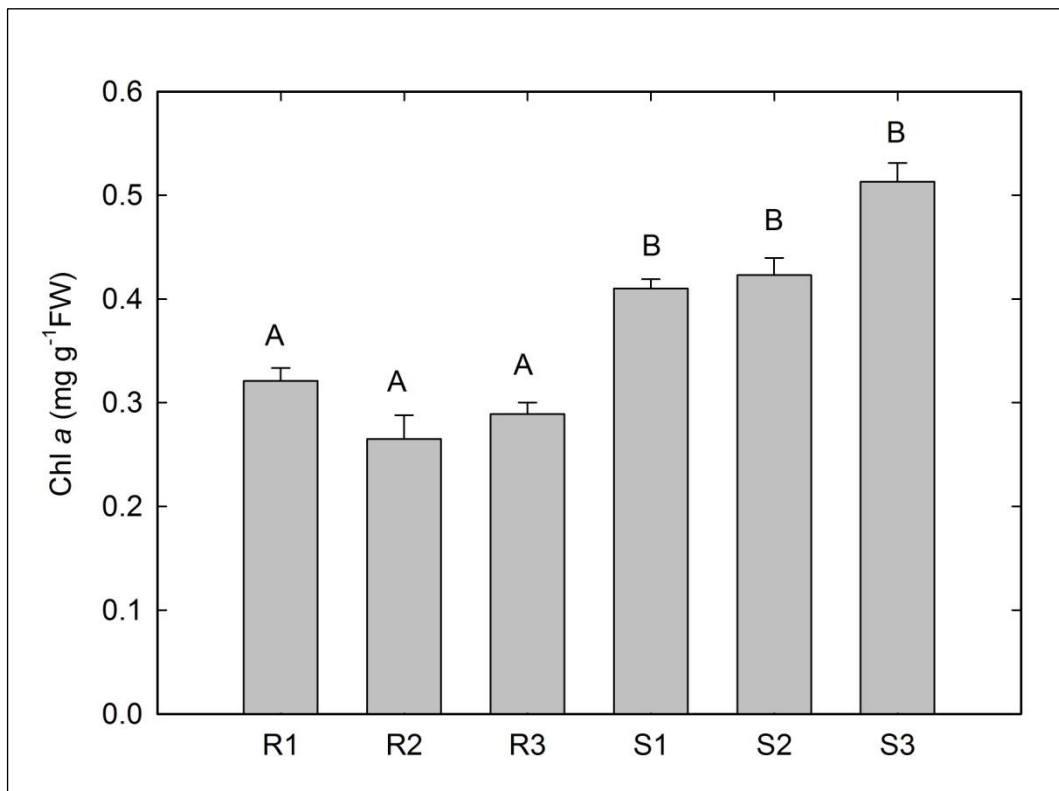
Station	Mean no. crystals (per 5 $\mu\text{m}^2$ )	Mean crystal length ( $\mu\text{m}$ )	Mean crystal width ( $\mu\text{m}$ )
R3	94 $\pm$ 6.65 <sub>(A)</sub>	1.30 $\pm$ 0.05 <sub>(A)</sub>	0.20 $\pm$ 0.01 <sub>(A,B)</sub>
R2	96 $\pm$ 7.47 <sub>(A)</sub>	1.44 $\pm$ 0.07 <sub>(A)</sub>	0.20 $\pm$ 0.01 <sub>(B)</sub>
R1	96 $\pm$ 8.20 <sub>(A)</sub>	1.43 $\pm$ 0.06 <sub>(A)</sub>	0.21 $\pm$ 0.01 <sub>(B)</sub>
S1	106 $\pm$ 4.76 <sub>(A)</sub>	1.80 $\pm$ 0.06 <sub>(B)</sub>	0.18 $\pm$ 0.01 <sub>(B)</sub>
S2	115 $\pm$ 8.74 <sub>(A)</sub>	1.54 $\pm$ 0.10 <sub>(A)</sub>	0.19 $\pm$ 0.01 <sub>(B)</sub>
S3	153 $\pm$ 6.31 <sub>(B)</sub>	1.52 $\pm$ 0.07 <sub>(A)</sub>	0.17 $\pm$ 0.01 <sub>(B,C)</sub>
ANOVA	$F = 8.430$ , $P =$ $<0.001^*$	$F = 5.662$ , $P =$ $<0.001^*$	$F = 3.881$ , $P =$ $<0.001^*$



**Fig. 6.4 a)** Images showing calcified surfaces of *P. pavonica* fronds sampled along the Vulcano CO<sub>2</sub> gradient, scale bar = 1 cm, white arrows indicate area where SEM analysis of aragonite crystals occurred **b)** SEM images of aragonite crystals on the surface of *P. pavonica* fronds sampled at R2 and S2 (showing thinner crystals), scale bar = 2 μm.

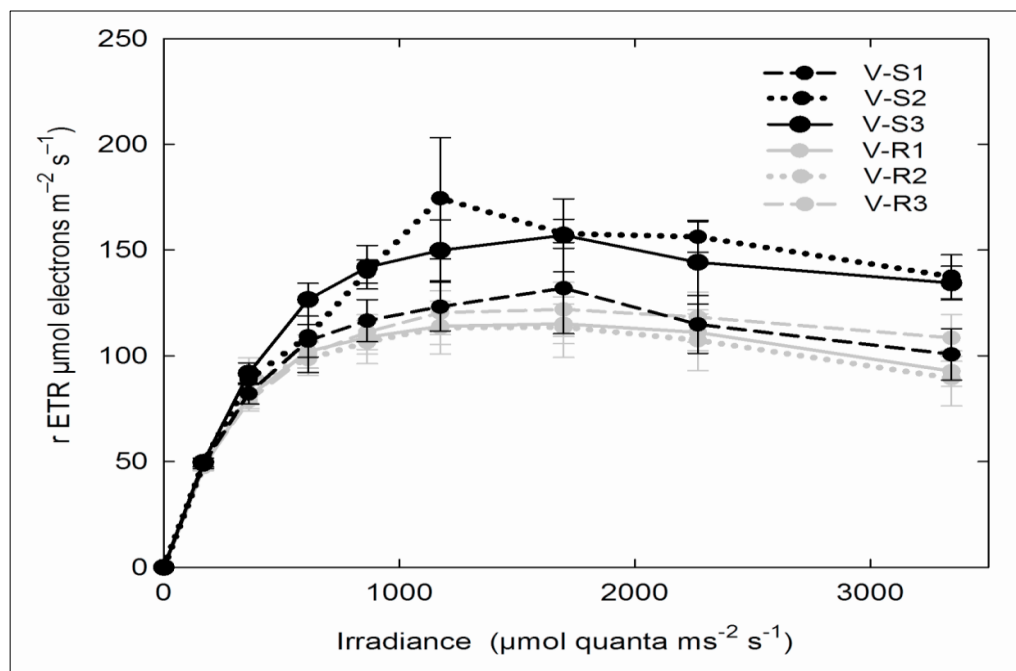


Chlorophyll *a* content in *P. pavonica* increased in the CO<sub>2</sub> enriched stations relative to the reference stations. There was a significant difference in Chl *a* from the frond samples analysed at S1, S2 and S3 (Fig 6.5; Kruskal Wallis:  $H_5 = 106.266$ ,  $P < 0.001$ ) compared with the reference stations R1-R3 (Dunn,  $P < 0.05$ ). No significant differences could be detected between the three reference stations (Dunn,  $P > 0.05$ ). Similar patterns were observed with Chl *c* content, CO<sub>2</sub> enriched stations contained more Chl *c* (S1 =  $0.05 \text{ mg g}^{-1} \text{ fw} \pm 0.002$ , S2 =  $0.06 \text{ mg g}^{-1} \text{ fw} \pm 0.002$ , S3 =  $0.07 \text{ mg g}^{-1} \text{ fw} \pm 0.003$ ) than those in the reference stations (R1 =  $0.04 \text{ mg g}^{-1} \text{ fw} \pm 0.002$ , R2 =  $0.04 \text{ mg g}^{-1} \text{ fw} \pm 0.004$ , R3 =  $0.04 \text{ mg g}^{-1} \text{ fw} \pm 0.003$ ). There was a significant difference in Chl *c* content detected between stations (Kruskal Wallis:  $H_5 = 60.531$ ,  $P < 0.001$ ),



**Fig. 6.5** Mean (+ SE) Chl *a* content in *P. pavonica* fronds along the Vulcano CO<sub>2</sub> gradient ( $n = 40$  per station). Means with the same letters are not significantly different ( $P > 0.05$ ).

There were no significant differences detected in the photochemical efficiencies ( $F_v/F_m$ ) between *P. pavonica* fronds between stations along the  $\text{CO}_2$  gradient (ANOVA:  $F_{(5,28)} = 0.682$ ,  $P > 0.05$ ). The differences observed in the photosynthetic responses ( $r\text{ETR}$ s) of *P. pavonica* to increased  $\text{CO}_2$  are presented in Table 6.2 and Figure 6.6. The  $r\text{ETR}$  max values were greater in the  $\text{CO}_2$  enriched stations (S2 and S3) compared with the reference stations. There was a significant difference in  $r\text{ETR}$  max values between stations (ANOVA,  $F_{(5,28)} = 2.756$ ,  $P < 0.05$ ). The greatest mean  $r\text{ETR}$ s values recorded at saturating irradiance ( $3344 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) were recorded at S2 and S3 ( $137.43 \mu\text{mol electrons m}^{-2} \text{s}^{-1} \pm 10.12$ ,  $134.45 \pm 7.97$  respectively). There was a significant difference between stations (ANOVA,  $F_{(5,28)} = 3.691$ ,  $P < 0.05$ ). There were no differences in  $r\text{ETR}$ s between stations under a subsaturating irradiance ( $360 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) (ANOVA  $F_{(5,28)} = 0.751$ ,  $P > 0.05$ ).



**Fig. 6.6** Rapid light curves of *P. pavonica* along the Vulcano  $\text{CO}_2$  gradient, showing the mean ( $\pm\text{SE}$ ) relative electron transport rates ( $r\text{ETR}$ ) per station ( $n = 5$  R3 + S3,  $n = 6$  for all other stations) at increasing irradiance.

**Table 6.2** Mean ( $\pm$ SE)  $rETR$  values across the Vulcano CO<sub>2</sub> gradient in May 2011 ( $n = 5-6$  per station) \* indicates sig value of one way ANOVA tests between stations ( $P < 0.05$ ). Means with the same letters are not significantly different ( $P > 0.05$ ).

$rETR$ values			
Station	Subsaturating irradiance (360 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ )	Supersaturating irradiance (3344 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ )	Max
R3	80.67 $\pm$ 5.84	108.46 $\pm$ 11.05 <sub>(A,BC)</sub>	123.47 $\pm$ 12.44 <sub>(A)</sub>
R2	87.96 $\pm$ 9.89	89.34 $\pm$ 12.05 <sub>(B)</sub>	115.72 $\pm$ 13.65 <sub>(A)</sub>
R1	78.65 $\pm$ 4.85	92.71 $\pm$ 9.29 <sub>(A,B,C)</sub>	116.42 $\pm$ 10.74 <sub>(A)</sub>
S1	82.19 $\pm$ 4.75	100.69 $\pm$ 13.01 <sub>(A,B,C)</sub>	134.25 $\pm$ 19.28 <sub>(A)</sub>
S2	88.74 $\pm$ 2.03	137.43 $\pm$ 10.12 <sub>(C)</sub>	182.04 $\pm$ 24.79 <sub>(B)</sub>
S3	91.74 $\pm$ 4.88	134.46 $\pm$ 7.97 <sub>(A,B,C)</sub>	163.01 $\pm$ 12.99 <sub>(A,B)</sub>
ANOVA	$F = 0.751, P = 0.592$	$F = 3.078, P = 0.024^*$	$F = 2.756, P = 0.038^*$

## 6.4 Discussion

This is the first study to investigate the *in situ* impacts of elevated CO<sub>2</sub> on calcification and photosynthesis in *Padina*. It is also the first *in situ* observation of the changes of both grazers and macroalgae along gradients of increasing concentrations of CO<sub>2</sub>. Along the rocky shores there was a reduction in sea urchin abundances alongside a proliferation of *P. pavonica* as CO<sub>2</sub> levels increased. It appears that the elevated CO<sub>2</sub> levels may influence algal-grazer dynamics as species assemblages change, causing profound structural and functional changes in rocky shore habitats. The findings from existing laboratory studies have raised concern for the survival of calcified macroalgae under conditions of high CO<sub>2</sub>. Indeed, previous investigations at CO<sub>2</sub> vent seeps have observed dramatic reductions in the abundance of calcified macroalgae (Hall-Spencer et

al. 2008; Martin et al. 2008; Fabricius et al. 2011; Porzio et al. 2011). The results from this investigation, however, indicate that some calcified algae may thrive as the oceans acidify, despite expected reductions in calcification. Here it is shown that *P. pavonica* can proliferate with CO<sub>2</sub> enrichment, appearing to thrive as a decalcified form in low pH as similarly recorded for some genera of fleshy macroalgae (Hall-Spencer et al. 2008; Connell and Russell 2010; Fabricius et al. 2011; Porzio et al. 2011).. Whilst significant increases were observed in *P. pavonica* abundance in September 2010, this was not the case in the other two sampling periods. Despite the fact that this may indicate that there were perhaps other, potentially sporadic, factors stimulating growth of the algae during the September 2010 period, it is key to point out that the abundance of *P. pavonica* did not decrease with elevations in pCO<sub>2</sub> across the three sampling periods, in stark contrast to other calcifying macroalgae along natural CO<sub>2</sub> gradients (Hall-Spencer et al. 2008; Porzio et al. 2011; Fabricius et al. 2011).

*Padina pavonica* calcification was negatively impacted by increasing CO<sub>2</sub>, the content of CaCO<sub>3</sub> significantly decreased with reductions in pH. This is consistent with other calcification studies on aragonitic macroalgae (Borowitzka & Larkum 1976; Price et al. 2011; Sinutok et al. 2011) and high magnesium calcitic macroalgae (Martin & Gattuso 2009; Semesi et al. 2009). Reductions in CaCO<sub>3</sub> content implies that herbivore defence mechanisms (and other benefits associated with calcification) will be compromised under low pH, causing an increase in grazing mortality. This was not, however, reflected *in situ*. Sea urchins are major predators of *Padina* spp. and their presence can cause significant reductions in the algae's abundance (Hereu et al. 2006). The absence of sea urchins in the CO<sub>2</sub> enriched areas may therefore explain the proliferation of *P. pavonica*, as it becomes released from the top-down control of these grazers. Similar effects of sea urchin removal have been observed in other *Padina* sp. populations (Sammarco et al. 1974) and across other phaeophyte assemblages (Liddell & Ohlhorst

1986; Leinaas & Christie 1996; Ling et al. 2010). Sea urchins are vulnerable to the effects of ocean acidification (Miles et al. 2007; Havenhand et al. 2008; Dupont et al. 2010) and therefore are an important co-variable to consider in understanding the responses of macroalgae to the future predicted changes in seawater carbonate chemistry. However, in this case, the absence of sea urchins at the CO<sub>2</sub> enriched areas may also be due to the lack of suitable habitat at these stations, specifically the increase in soft bottom substrata relative to hard bottom substrata. Despite this, similar reductions in sea urchins were also noted along other vent gradients in the Mediterranean (Hall-Spencer et al. 2008) and Papua New Guinea (Johnson et al. 2012b), which were not subjected to the same physical changes in benthos, indicating that the responses of sea urchins observed here may still be an accurate representation of future change.

Increased productivity with elevated CO<sub>2</sub> may also account for the proliferation of *P. pavonica* at low pH. Laboratory studies of other calcified macroalgae have revealed declines in photosynthetic pigments in high CO<sub>2</sub>/low pH treatments (Gao & Zheng 2010; Sinutok et al. 2011) which are indicative of chlorophyll degradation, a reduction in photosynthetic unit size and/or a reduction in PSII reaction centres (Sinutok et al. 2011). These findings, however, show the opposite. Chl *a* and Chl *c* content in *P. pavonica* was greater in the CO<sub>2</sub> enriched stations indicating an increase in photosynthetic capacity under conditions with high CO<sub>2</sub>. A possible cause for the lower Chl *a* content in fronds from ambient pH may be due to the higher CaCO<sub>3</sub> contents relative to those in low pH, which have undergone decalcification. The relative differences in live tissue weight between calcified and non-calcified fronds may therefore influence the final Chl *a* contents as they are expressed in  $\mu\text{g mg}^{-2}$ . In this case however, CO<sub>2</sub> levels appear to be a more likely cause for the variations as fronds from S2 and S3 shared similar CaCO<sub>3</sub> contents yet Chl *a* content was higher in S3 relative to S2.

It has been speculated that pH stress may negatively impact photosynthetic performance through the disruption of the CO<sub>2</sub> accumulating pathway at the site of Rubisco, or interference with electron transport (Anthony et al. 2008). This has been supported though laboratory experiments with *Halimeda* spp. which have demonstrated declines in photosynthetic efficiency (maximum quantum yield; Fv/Fm) (Sinutok et al. 2011) and response ( $rETR_{max}$ ) (Price et al. 2011) under elevated CO<sub>2</sub>. In contrast, there was no significant effect of pH on photosynthetic efficiency (Fv/Fm) observed along the CO<sub>2</sub> gradient. Furthermore, a significant effect on the *in situ* photosynthetic responses of *P. pavonica* was observed with CO<sub>2</sub> enrichment (increases in  $rETR_{max}$  and mean  $rETR_{max}$  at supersaturating irradiance). However, it should be noted here that the irradiance values corresponding to the supersaturating values (3344  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) are unrealistically high in these particular habitats. Whilst some species of *Padina* are thought to possess carbon concentrating mechanisms (Raven et al. 2002a; Enríquez & Rodríguez 2006) *P. pavonica* is not believed to be carbon-saturated in ambient seawater and, at times, has been shown to utilise more inorganic carbon if it is provided as CO<sub>2</sub> (Einav et al. 1995). The positive photosynthetic response of *P. pavonica* to CO<sub>2</sub> enrichment therefore indicates a direct enhancement of carbon fixation across the gradient. Increased photosynthetic activity at high CO<sub>2</sub> has also been observed in other calcified macroalgae (Reiskind et al. 1988; Gao et al. 1993a; Semesi et al. 2009) and non-calcified macroalgae (Gao et al. 1991, 1993b; Kübler et al. 1999; Connell & Russell 2010; Russell et al. 2011b). As the photosynthetic measurements are from one season it cannot be assessed whether the photosynthetic responses of *P. pavonica* vary seasonally however these data provide an important snapshot of responses along gradients of increasing CO<sub>2</sub>.

It has been established that photosynthesis can stimulate calcification in algae (Borowitzka 1982; Gattuso et al. 1999). Okazaki et al. (1986) showed that aragonite

deposition in *Padina* begins in the intracellular space formed by the infolded apical margin of the thallus and, since chloroplasts also occur in this region, the authors suggest that this may indicate a relationship between the initiation of calcification and photosynthesis. Photosynthesis-induced calcification has also been demonstrated in the inter-utricular spaces of the aragonitic genus *Halimeda* (Borowitzka 1989). Increased  $\text{CaCO}_3$  dissolution in lower pH may, therefore, be offset by increased photosynthesis in those regions with chloroplasts. This may help to explain why, even in the lowest pH conditions, *P. pavonica* was still able to calcify, seemingly from the enhancement of photosynthesis under high levels of  $\text{CO}_2$ . Alternatively, the high pH variability in the vent zone, caused by transient exposure to ambient pH conditions (i.e periods of high winds increasing the mixing of vent waters with surrounding high pH seawater), has the potential to buffer the effects of acidification by relieving physiological stress (Hoffmann et al. 2011).

There is a lack of laboratory evidence of the effects of low pH on *Padina* spp. calcification to confirm whether decreased calcification is a direct response to reduced pH as opposed to, for example, the reduced grazing pressure in this *in situ* experiment. An investigation of Caribbean *Padina* sp. (Lewis et al. 1987) however, revealed that in heavily grazed areas the algae existed in the form of an uncalcified turf whereas in areas of low grazing activity it grew as calcified, foliose blades. The fact that these algae still choose to calcify when grazing intensity is low suggests that the reduced calcification recorded in this study may indeed be a direct response to lowered pH and not the changes in grazing pressure. It has been suggested that calcium carbonate crystal morphology and abundance may be associated with seawater chemistry: thinner, more abundant crystals have been shown to indicate reduced pH conditions as crystallisation events are thought to be initiated and terminated more frequently (Robbins et al. 2009; Sinutok et al. 2011). Over the thin calcified band in the apical region of *P. pavonica*

fronds in the CO<sub>2</sub> enriched station S3, there was a higher abundance of aragonite crystals recorded than in the reference stations and a decreasing trend of crystal width was observed with increasing levels of CO<sub>2</sub>. These results therefore support the theory of pH-dependent changes in calcium carbonate crystal morphology and deposition in calcified macroalgae. The implications of changes in *Padina* spp. bio-calcification on thallus rigidity, dissolution rates and overall sediment budgets however, need to be investigated further.

These findings indicate that, despite experiencing conditions that reduce calcification, *Padina* could in fact be one of the ecological winners of ocean acidification. This may be explained by a combination of factors; reduced sea urchin predation (and presumably other calcareous predators such as gastropods), increased photosynthetic capacity and performance and optimised energy reallocation following a reduction in carbon limitation. This work adds to evidence for proliferation of phaeophytes in a high CO<sub>2</sub> world (Hall-Spencer et al. 2008; Connell & Russell 2010; Diaz-Pulido et al. 2011; Russell et al. 2011, see Appendix I b & c) which has potentially profound consequences for the structure, function and resilience of a variety of benthic ecosystems globally (Russell et al. 2009; McManus & Polsenberg 2004; Harries et al. 2007). This study has highlighted large differences in the impacts of CO<sub>2</sub> enrichment between *Padina* and other calcified species, highlighting the importance of studying a wide range of genera before more accurate wider scale predictions concerning the impacts of ocean acidification on benthic calcified macrophytes can be formulated. It has also demonstrated that the responses of *Padina* to CO<sub>2</sub> enrichment is complex and requires an *in situ*, ecosystem based approach, incorporating multi-species interactions, highlighting the importance of natural CO<sub>2</sub> gradients as a valuable tool in the study of ocean acidification.



## Chapter 7: General Discussion

Ocean acidification is an emerging threat to marine ecosystems globally (Kleypas et al. 2006; Doney et al. 2009a,b; Kroeker et al. 2010). This process, caused by rising atmospheric CO<sub>2</sub>, is already underway (Solomon et al. 2007) and will accelerate under the predicted increases in atmospheric CO<sub>2</sub> over coming decades (IPCC 2007). There is, however, still a high level of uncertainty about how marine systems will be affected by these rapid changes in seawater chemistry. Progress in the understanding of the possible impacts of ocean acidification on marine biota is limited by the scarcity of information on responses at the community and ecosystem level (Gazeau 2011; Wernberg et al. 2012). Natural CO<sub>2</sub> gradients are beginning to reveal the ecological shifts that can be expected to occur with globally increasing atmospheric CO<sub>2</sub> in both temperate (Hall-Spencer et al. 2008; Porzio et al. 2011) and tropical benthic ecosystems (Fabricius et al. 2011; Uthicke & Fabricius 2012). Volcanic vent systems are useful as they can reveal communities of interacting organisms that are resilient to long term increases in CO<sub>2</sub> levels. They retain natural pH variability and acidify volumes of water on scales large enough to integrate ecosystem processes such as competition and predation. This thesis used a temperate volcanic CO<sub>2</sub> gradient to predict the impacts of ocean acidification on benthic algal assemblages. A comparison was also made with tropical vent systems (Johnson et al. 2021b). We need to improve our understanding of the responses of marine primary producers since these support food webs, drive biogeochemical cycles and profoundly affect the structure and function of benthic habitats.

## 7.1 Discussion of main findings and implications for marine systems

### 7.1.1 Microphytobenthic assemblages

In near-shore shallow water environments, the microphytobenthos forms an important functional component, exhibiting high areal rates of photosynthesis and contributing significantly to primary production, often exceeding that of the phytoplankton in the overlying waters (MacIntyre et al. 1996). Surprisingly, however, experimental evidence concerning the effects of elevated CO<sub>2</sub> on benthic microalgae remains very limited. To date, most studies that have investigated the impacts of elevated CO<sub>2</sub> on photoautotrophs have focused on phytoplankton (e.g. Tortell et al. 2002, 2008; Kim et al. 2006; Fu et al. 2007; Trimborn et al. 2009; Nielson et al. 2010). Our knowledge of the responses of benthic microalgae and microphytobenthic assemblages to ocean acidification is limited to a few recent laboratory and mesocosm experiments (Torstensson et al. 2011; Witt et al. 2011; Hicks et al. 2011). There is likely much to learn about the responses of photoautotrophs from observations in areas that are naturally enriched with CO<sub>2</sub> (Liu et al. 2010). The research for this PhD thesis appears to be the first to investigate the effects of elevated CO<sub>2</sub> on benthic microalgae assemblages *in situ*, thereby addressing some important gaps in our knowledge.

Table 7.1 summarises the main findings of investigations which studied the responses of a variety of microphytobenthic assemblages to CO<sub>2</sub> enrichment. This indicates that the responses of microphytobenthic assemblages to increasing CO<sub>2</sub> are largely positive, suggesting that many benthic microalgal taxa are not likely to be under threat from the effects of ocean acidification. Indeed, some appear to gain substantial benefits from increasing CO<sub>2</sub> as the photosynthetic biomass of most of the assemblages studied significantly increased in high CO<sub>2</sub> conditions. This is likely to have profound

consequences for coastal ecosystems as it indicates that primary productivity is likely to be enhanced with increasing concentrations of atmospheric CO<sub>2</sub> and, according to emission projections (Nakićenović & Swart 2000; Feely et al. 2009), these changes are likely to occur from around the midpoint of this century in some assemblages (Johnson et al. 2012a). Further support for CO<sub>2</sub> enhancement of productivity in benthic habitats comes from a recent investigation into the symbiotic microalgae, *Symbiodinium* spp. along a CO<sub>2</sub> gradient off Vulcano Island, Sicily, (Suggett et al. 2012). Elevations in CO<sub>2</sub> resulted in increased gross photosynthesis rates in these symbiotic algae, which in turn, supported enhanced productivity in the host anemone populations.

On both natural and artificial surfaces, biofilm production increased substantially in high CO<sub>2</sub> conditions. These findings have since been supported by a similar biofilm study along the same CO<sub>2</sub> gradient which found that the content of uronic acids, a significant component of the extracellular polymeric substances in biofilms, increased with CO<sub>2</sub> enrichment (Lidbury et al. 2012). The colonisation of newly exposed substrata by microbial biofilms is a ubiquitous process in the marine environment. Enhancement of photoautotrophic biofilms under elevated CO<sub>2</sub> therefore has the potential to promote primary productivity (Hawkins et al. 1992; Bustamante et al. 1995) and increase the carbon source of food webs (Nagarkar et al. 2004; Doi et al. 2008) across a variety of marine benthic habitats.

Furthermore, increased biomass of photoautotrophic biofilms has wider ecological significance in relation to the potential impacts of ocean acidification on marine biota, for example, grazing gastropods require more energy to sustain increased calcification rates under conditions of high CO<sub>2</sub> (Rodolfo-Metalpa et al. 2011). There are also, however, potentially negative consequences of enhanced biofilm production. For marine industries, there may be considerable economic repercussions associated with the

potential increase in biofilm development and persistence under high CO<sub>2</sub> (Yebra et al. 2004; Schultz 2007).

The photosynthetic standing crop of the microphytobenthos associated with sandy sediments was also highest at elevated levels of CO<sub>2</sub>. This has important ecological and biogeochemical implications for coastal permeable sediments where the microphytobenthos constitutes the main source of organic matter (MacIntyre et al. 1996; Nelson et al. 1999). It may also have significant consequence for the dynamics of these shallow coastal systems, as the microphytobenthos is known to regulate the content and flux of regenerated nutrients and oxygen in sediments (Sundbäck et al. 1991; Cahoon et al. 1999) and influence biogeochemical processes (Shum & Sundby 1996; Boudreau et al. 2001). Microphytobenthic assemblages are also known to act as modifiers of coastal sediments, in particular, through their role in stabilising sediments (Krumbein et al. 1994). Therefore, stimulation of these assemblages under high CO<sub>2</sub> may have positive effects for those coastal habitats facing the increasing threat of global climate change-induced coastal erosion (Nicholls et al. 2007).

We presently know very little of the effects of elevated CO<sub>2</sub> on the microphytobenthos of muddy sediments. Evidence so far indicates that microphytobenthic assemblages in these habitats are not CO<sub>2</sub> limited, as they have not responded to elevations in mesocosm experiments (Hicks et al. 2011). Therefore the predictions based on the responses observed in this study may not be applicable for all types of coastal sediments. The contrasting responses between these two sediment types may be due to the fact that permeable sands and cohesive mud habitats produce different environmental conditions for the microphytobenthos they support (Kuhl & Jørgensen 1994; Paterson & Hagerthey 2001; Jesus et al. 2009). In particular, we may expect that the microphytobenthos have physiologically acclimatised to different background CO<sub>2</sub>

concentrations due to crucial differences in advective pore-water transport from the water column between the two types of sediment (Huettel & Webster 2001; Cook & Røy 2006) and this may influence microphytobenthic responses to elevated CO<sub>2</sub>.

The effect of elevated CO<sub>2</sub> on endolith activities is a prime research topic (Tribollet 2008). This is due to the various biological and biogeochemical implications associated with enhancement of these assemblages under high concentrations of CO<sub>2</sub>, in particular their potential ability to buffer seawater under ocean acidification scenarios. Despite lack of statistical confirmation, there is some indication from the data collected here that endolithic assemblages may respond positively to elevations in CO<sub>2</sub>. Alongside previous studies (Halley et al. 2005; Tribollet et al. 2009) this indicates that elevations in *p*CO<sub>2</sub> may lead to higher rates of biogenic dissolution. Current estimates indicate that, despite the predicted increases in the chemical dissolution of carbonate sediments with lowered seawater pH, this chemical dissolution will not produce enough alkalinity to buffer the predicted drop in surface water pH over the next 100 years (Feely et al. 2004). These estimates are, however, based on biogeochemical models which do not take into account the effects of rising CO<sub>2</sub> on the biogenic dissolution by endolithic algae (Tribollet et al. 2009). This emphasizes the importance of repeating these investigations along natural CO<sub>2</sub> gradients for improving our understanding of the potential consequences of CO<sub>2</sub>-induced stimulation of endolithic assemblages on biogeochemical cycles, ocean acidification feedback mechanisms and the resilience of coral reef ecosystems

**Table 7.1** Summary of the responses of various microphytobenthic (MPB) assemblages to increasing levels of  $p\text{CO}_2$ . Legend continues overleaf

MPB assemblage	Habitat	Photosynthetic biomass (Chl <i>a</i> )	Benthic diatom abundance	Cyanobacteria abundance	Chlorophyte abundance
<b>General Periphyton</b>	Surface of artificial (perspex) substrata	↑	↑ *	↔ N.B. Station with the highest $\text{CO}_2$ exposure S3 not sampled	—
<b>Epilithic</b>	Surface of igneous rock	↑	↑ *	↑ / ? (phycobilin fluorescence, interference from rhodophyta)	—
<b>Epipsammic</b>	Surface of sand grains	↑ (combined with epipellic fraction of sediment)	↑	—	—
<b>Epipellic</b>	Within sandy sediments, motile between sand grains	↑ (combined with epipsammic fraction of sediment)	↑ * (epifluorescence microscopy) ↑ (light microscopy)	↑	—
<b>Endolithic</b>	Within aragonite blocks (stations S1 & S2 only)	↔ ↓ / ↓	N/A	↔	↔
	Within live gastropod shells	↓ / ↓		↔	↓

### Table 7.1 Legend



Increased concomitantly with elevations in  $p\text{CO}_2$  concentrations (linear response)



Overall increase in high  $\text{CO}_2$  stations relative to reference station (but non-linear response)



Increased under the highest  $\text{CO}_2$  exposure only ( $>1,400 \mu\text{atm}$ )



No significant change recorded



Decreased under the highest  $\text{CO}_2$  exposure only ( $>1,400 \mu\text{atm}$ )



Overall decrease in high  $\text{CO}_2$  stations relative to reference station (but non-linear response)



Decreased concomitantly elevations in  $p\text{CO}_2$  concentrations (linear response)

—

Not investigated

\*

Shifts in community composition observed with elevations in  $\text{CO}_2$

### 7.1.2 Benthic Diatoms

Table 7.1 reveals that the abundance of benthic diatoms increases in response to elevated CO<sub>2</sub> across a variety of substrata. This supports the findings from several other studies which have documented positive effects of CO<sub>2</sub> enrichment on diatoms (Tortell et al. 2002, 2008; Feng et al. 2009; Sun et al. 2011). These positive responses may be explained by the notion that some diatoms will benefit from increasing CO<sub>2</sub> through a reduction in the energetic costs of their carbon concentrating mechanism, optimising resource allocation (Beardall & Giordano 2002; Rost et al. 2008; Trimborn et al. 2009; Hopkinson et al. 2011). This hypothesis is based on our current level of understanding on the microalgal responses to CO<sub>2</sub>, however the literature is heavily biased towards oceanic microalgae, we may expect benthic microalgae to differ in their physiological requirements and this should be considered further as this field of research progresses. It is also important to discuss the potentially confounding factor of nutrient concentrations. The slightly elevated nitrite concentrations (0.01 µM higher at stations S2 and S3) appear to be too low to sustain the substantially enhanced abundances observed across these habitats. There was an increasing trend of silicate concentrations along the CO<sub>2</sub> gradient. However, as discussed in Chapter 3, it is likely that the silicate concentrations at the reference stations were not low enough to limit diatom growth. Furthermore, diatom abundance, in a range of different benthic assemblages, often increased concomitantly with *p*CO<sub>2</sub> (Table 7.1) and increased significantly between stations which had similar nutrient conditions (i.e. between R2 and S1, and between S2 and S3). Thus it appears that the primary response of diatoms along this gradient is to CO<sub>2</sub>.



Considering the significant role diatoms play in ocean primary production (Falkowski et al. 1998; Field et al. 1998) and biogeochemical cycles (Tréguer et al. 1995; Nelson et al. 1995) there are likely to be profound and widespread consequences of near-future, CO<sub>2</sub> induced enhancement of diatom populations for ocean systems. Changes in marine primary production can significantly alter the cycling and storage of carbon and other bioactive elements in the oceans, leading to important biogeochemical and climatic feedbacks (Riebesell 2004). That benthic diatoms thrived under high CO<sub>2</sub> conditions in this study lends support to a model that shows that doubling atmospheric CO<sub>2</sub> could result in up to a two-fold increase in photosynthetic carbon fixation by marine and freshwater algae (Schippers et al. 2004). Increased diatom growth and carbon fixation rates have the potential to accelerate carbon sequestration from the atmospheric CO<sub>2</sub> reservoir via the ‘biological pump’, this, in turn, may provide a negative feedback on increased atmospheric CO<sub>2</sub> (Tortell et al. 2008). This, however, assumes that photosynthesis is not limited by other factors such as nutrients and light, which may also alter under global climate change (Feng et al. 2009). For example, rising sea temperatures may cause surface ocean stratification to intensify. This would impede the mixing of ocean layers, preventing nutrients from deeper layers reaching the surface and affecting the light regime to which phytoplankton are exposed (Rost et al. 2008).

Potential CO<sub>2</sub>-dependent productivity increases have wide ranging biogeochemical consequences. However, the magnitude of future change in carbon sequestration will depend on the responses of individual microbial assemblages to increasing CO<sub>2</sub> levels (Hare et al. 2007). Whilst this thesis has recorded significant enhancement in benthic diatom growth in microphytobenthic assemblages, field incubation experiments have shown little or no effect of CO<sub>2</sub> manipulations on overall growth rates or primary

productivity of mixed phytoplankton assemblages (Tortell et al. 2002; Hare et al. 2007). Furthermore, there have been several studies suggesting that pelagic diatom responses to CO<sub>2</sub> are negative or small (Tortell et al. 1997; Burkhardt et al. 1999; Crawford et al. 2011).

The explanation for the discrepancies in responses between benthic and pelagic microalgae may be attributed to differences in inorganic carbon limitation.

Photosynthesis by pelagic algae is generally not considered to be limited by CO<sub>2</sub> availability in the ocean (Raven 1997) and therefore may explain the small effects of elevated CO<sub>2</sub> on the phytoplankton assemblages studied. Microphytobenthic assemblages, on the other hand, have extremely high volume-specific rates of primary production relative to pelagic algae, and their position at the surface-water interface results in solute transport to and from the cell becoming limited by diffusion across the benthic boundary layer, particularly in sediments (Jørgensen 2001), and presumably within thick biofilms. These factors result in extreme conditions within the photosynthetic layer of microphytobenthos including pH values in excess of 9 (de Jong et al. 1988), caused by CO<sub>2</sub> assimilation. Under these conditions, CO<sub>2</sub> assimilation may be significantly reduced even in microalgae with a high affinity for inorganic carbon (Raven & Johnston 1991). Further experimentation has provided strong evidence to suggest that a variety of microphytobenthic assemblages (cultured diatoms films, coral reef epilithic algae, and the microphytobenthos in sandy poorly flushed sandy sediments) may experience inorganic carbon limitation under present atmospheric CO<sub>2</sub> concentrations (Admiraal et al. 1982; Larkum et al. 2003; Cook & Roy 2006). This may explain the observed increases in benthic diatom abundance under CO<sub>2</sub> enrichment across the range of habitats studied here. The discrepancies in the responses observed in

phytoplankton (i.e combination of both positive and neutral), however, still remains unclear. It seems likely that different responses to changes in CO<sub>2</sub> across similar habitats could be due to particular CCM operating in each species, leading to species-dependent CO<sub>2</sub> related effects and/or differences in the control of carbonate system between culture experiments.

Benthic diatoms appear to exhibit a non-uniform response to CO<sub>2</sub> as the composition of assemblages was found to alter significantly as CO<sub>2</sub> levels increased across a variety of substrata. This is likely due to being affected by taxa-specific differences in the regulation and kinetics of inorganic carbon uptake (Rost et al. 2003; Trimborn et al. 2009). Elevations in CO<sub>2</sub> may alter the competitive abilities among different size classes of diatoms as shifts in dominance to large, chain forming genera were recorded, as similarly observed in phytoplankton assemblages (Tortell et al. 2008). Comparable CO<sub>2</sub> induced shifts in species composition have also been observed across other photoautotrophic assemblages (Tortell et al. 2002, 2008; Fu et al. 2007; Russell et al. 2009; Trimborn et al. 2009; Connell & Russell 2010; Porzio et al. 2011; Witt et al. 2011). This study therefore contributes to an increasing body of evidence that rising CO<sub>2</sub> emissions may lead to structural and functional changes in a wide variety of marine and coastal habitats. Alterations in the taxonomic composition of benthic diatom assemblages would be expected to affect the structure and function of overlying benthic communities, since diatoms play important roles in settlement of marine biota and influence the outcomes of benthic succession (Meadows & Williams 1963; Huang & Boney 1984; Thompson et al. 1998). Further observations and experimentation is required to document the differences in secondary succession between benthic habitats exposed to different concentrations of CO<sub>2</sub>.

### 7.1.3 Benthic Cyanobacteria

The responses of benthic cyanobacteria to increasing CO<sub>2</sub> levels observed in this study are very different from those reported for benthic diatoms (Table 7.1). The benthic forms of cyanobacteria off Vulcano Island, Sicily, were less sensitive to changing CO<sub>2</sub> than the benthic diatoms. This may be explained, in part, by reports of their highly efficient CCM, thought to be one of the most effective of any photosynthetic organism (Badger & Price 2002). The findings from volcanic vents at Vulcano do not support the notion that elevations in CO<sub>2</sub> are beneficial to cyanobacteria through down-regulation of their high-cost CCM. However, it is also possible that the oligotrophic conditions of the Mediterranean study site (Kletou & Hall-Spencer 2012) limit the potential effects of CO<sub>2</sub> enhanced productivity in this group of microalgae, this may be indicated by the proliferation of cyanobacteria at S3, where some nutrients were elevated. This could help explain the contrasting results of laboratory studies which have shown strong positive responses of cyanobacteria under nutrient replete conditions (Barcelos e Ramos et al. 2007; Hutchins et al. 2007; Levitan et al. 2007; Fu et al. 2008b; Kranz et al. 2009). Light intensities have also been found to modify the responses of cyanobacteria (*Trichodesmium* spp.) to pCO<sub>2</sub> (Kranz et al. 2010; Levitan et al. 2010). In particular, CO<sub>2</sub> effects on *Trichodesmium* spp. were found to diminish at higher light intensities such as those which prevail close to the ocean's surface. As the assemblages studied here were sampled from shallow water (< 0.5 m), light levels will be relatively high compared to oceanic assemblages. This may offer further explanation for the differences in cyanobacteria sensitivity observed between these benthic assemblages and oceanic taxa studied in previous laboratory studies.

At the station where positive responses of cyanobacteria were reported (S3) it is not possible to confidently isolate the effects of high CO<sub>2</sub> from nutrient enrichment, indeed it may be due to a combination of both, and the lack of CO<sub>2</sub> enrichment studies on benthic cyanobacteria further complicates the interpretation of this response. It appears that there may be large differences in the sensitivities and responses of cyanobacteria to CO<sub>2</sub> between benthic and pelagic habitats. The contrasting responses of various species of cyanobacteria to ocean acidification is thought to be attributed to differences in ecological strategies (Czerny et al. 2009), and species/strain specific differences in CCM (Lu et al. 2006; Fu et al. 2007). Further research, involving a wide variety of cyanobacterial genera from both benthic and oceanic habitats investigated under a range of CO<sub>2</sub> and nutrient conditions is required, in order to advance our understanding about the effects of CO<sub>2</sub> enrichment on this group of microalgae.

The majority of bloom-forming cyanobacteria species experiments indicate that increasing levels of CO<sub>2</sub> could significantly alter nitrogen inputs to the ocean and profoundly affect global biogeochemical cycles over the course of this century (Hutchins et al. 2009). Based on the present study however, the ecological and biogeochemical consequences of near-future ocean acidification scenarios on benthic cyanobacteria appear to be relatively small or negligible, under nutrient limitation in coastal temperate systems at least.

#### *7.1.4 Macroalgal assemblages*

Sub-tidal investigations along the Vulcano CO<sub>2</sub> gradient revealed dramatic alterations in macroalgal assemblages (Appendix I). These findings contribute to a growing body of

evidence collected from other vent sites that indicates elevations in CO<sub>2</sub> are likely to perturb the structure and function of shallow water benthic habitats over the course of this century (Hall-Spencer et al. 2008; Fabricius et al. 2011; Porzio et al. 2011; Graziano et al. submitted; Suggett et al. 2012). Macroalgal community shifts, for example, can alter the abundance and diversity of herbivores in coastal ecosystems (Benedetti-Cecchi et al. 2001). The potential effects of ocean acidification-altered community structure on ecosystem functioning are, however, presently unclear, especially since the effects of elevated CO<sub>2</sub> levels on organism interactions have only recently begun to be addressed (Diaz-Pulido et al. 2011; Doropoulos et al. 2012).

Chapter 6 (Johnson et al. 2012b) however, appears to be the first *in situ* demonstration of the effects of elevated CO<sub>2</sub> on grazer-algal population dynamics. Along a rocky shore CO<sub>2</sub> gradient in Sicily there were dramatic reductions in sea urchin abundance alongside a proliferation of *Padina pavonica* as CO<sub>2</sub> levels increased. These shifts in benthic community structure were strikingly similar to those documented at another CO<sub>2</sub> vent site in Italy, which recorded reductions in sea urchins whilst several phaeophytes thrived in seawater with high CO<sub>2</sub> levels (Hall-Spencer et al. 2008). In both systems the elevated CO<sub>2</sub> levels appear to be influencing algal-grazer dynamics as species assemblages change, causing profound ecological changes in rocky shore habitats. Indeed, the structure and function of ecosystems under future conditions is likely to represent changes to the balance between productivity and consumption (Connell et al. 2011). These changes in benthic community composition were detected at predicted near-future median pCO<sub>2</sub> levels of ~500 µatm (median pH 8.08). Investigations of sub-tidal epilithic algal communities revealed dramatic shifts in the composition of two functionally important benthic algal components, crustose coralline algae and turf algae

also at  $p\text{CO}_2$  levels of  $\sim 500 \mu\text{atm}$ . According to climate change predictions (IPCC 2007), these findings therefore indicate that we may begin to witness benthic ecological shifts occurring from around the midpoint of this century. This may have profound consequences for the structure and function of shallow water habitats due to associated changes in benthic flora and fauna recruitment, productivity and structural habitat stability. The expansion of phaeophytes and turf algae, along with an enhancement in the competitive strength of macroalgae over corals under ocean acidification scenarios (Diaz-Pulido et al. 2011) threatens the resilience of coral reef ecosystems by facilitating the shift from coral dominated to algae dominated reefs (McManus & Polsenberg 2004).

Consistent with other calcification studies on macroalgae (Martin & Gattuso 2009; Semesi et al. 2009; Price et al. 2011; Sinutok et al. 2011), calcification in *P. pavonica* was negatively impacted by increasing  $\text{CO}_2$  levels. However, in contrast to these studies, this did not affect the success of *P. pavonica* in low pH environments. Indeed, the findings presented in Chapter 6 suggest that *Padina* spp. could, in fact, be amongst the ecological winners under ocean acidification scenarios, alongside a range of fleshy macroalgae (Kübler et al. 1999; Porzio et al. 2011; Raven et al. 2011). The success of decalcified *P. pavonica* at low pH may be explained by a combination of factors including; reduced sea urchin predation, increased photosynthetic capacity and performance, and optimised energy reallocation following a reduction in carbon limitation. This illustrates the importance of considering the indirect effects that occur within multispecies assemblages when attempting to predict the consequences of ocean acidification on marine biota and communities (Hale et al. 2011).

Large differences in the tolerance of CO<sub>2</sub> enrichment between *P. pavonica* and other calcified species have been made apparent by this thesis. This highlights the importance of studying a wide range of genera to better inform global predictions of the impacts of ocean acidification on marine ecosystems (Russell et al. 2011b). This thesis has also demonstrated that the response of *P. pavonica* to CO<sub>2</sub> enrichment is complex and potentially multi-factorial. An *in situ*, ecosystem based approach, incorporating multi-species interactions and predator-prey dynamics, provides more accurate insights into the responses of marine organisms than single-species laboratory manipulations, highlighting the importance of natural CO<sub>2</sub> gradients as a valuable tool in the study of ocean acidification. An investigation along comparable CO<sub>2</sub> gradients off Papua New Guinea (Johnson et al. 2012b) supported the findings from Chapter 6. Along two CO<sub>2</sub> vent gradients in Papua New Guinea, similar reductions in the calcification of *Padina australis* were recorded and this species was also found to proliferate at low pH, alongside reductions in sea urchin abundances. This constituted the first study to directly compare ecological changes along temperate and tropical CO<sub>2</sub> vent gradients. The striking similarities found in the responses of *Padina* spp. and sea urchin abundance at several vent systems increases confidence in these predictions over a large geographical range.

## **7.2 Limitations of research**

The limitations in using CO<sub>2</sub> vent systems as proxies for future ecological change (e.g. high pH variability, immigration of organisms from ambient conditions) were discussed in Chapter 2. There are, however other potential limitations of the Vulcano study that



need addressing here. The control of the carbonate system in observational, *in situ* studies is relatively limited compared to laboratory experiments and it is more difficult to incorporate and control the modulation of other environmental factors (Barry et al. 2010). Numerous other environmental variables such as nutrients, temperature and light will modify the responses of marine phototrophs to CO<sub>2</sub>. For instance, increasing CO<sub>2</sub> may interact with spatial and temporal variations in nutrient concentrations to create regionally different responses of algal assemblages to CO<sub>2</sub> enrichment (Russell et al. 2009). Some coastal ecosystems in the Mediterranean Sea are extremely oligotrophic with respect to the rest of the world oceans, with nutrient levels that are similar to mid ocean gyres (Crispi et al. 1998; Kletou & Hall-Spencer 2012). This therefore needs to be considered when scaling predictions made in this study to different ecosystems, as the CO<sub>2</sub> elevations observed here may have different effects compared with more eutrophic waters.

Multiple environmental stressors need to be considered when predicting the responses of ecosystems to ocean acidification. Many recent ocean acidification studies now address the potential modulation of other environmental variables and potential interactions arising from global climate change (Rodolfo-Metalpa et al. 2010, 2011; Chen & Gao 2011; Russell et al 2011a; Sinutok et al. 2011; Hoogstraten et al. 2012). Due to the difficulties of controlling environmental variables in observational CO<sub>2</sub> vent studies this thesis was limited to studying the effects of high CO<sub>2</sub> (with the exception of slightly elevated nutrients at S3). This highlights the importance of using field studies in conjunction with laboratory experiments which are able to manipulate a variety of environmental parameters.

### 7.3 Summary and direction for future research

Ocean acidification is one of the most complex challenges facing twenty first century research (Wick & Roberts 2012). This thesis contributes to a scant, but growing body of evidence concerning the long term effects of elevated CO<sub>2</sub> on benthic photoautotrophic assemblages. It is clear from these findings that along gradients of pH/CO<sub>2</sub> assemblages of benthic algae alter significantly. Under naturally high *p*CO<sub>2</sub> conditions that emulate near-future ocean acidification conditions, the photosynthetic biomass of a variety of microphytobenthic assemblages was found to be significantly enhanced, important ecological shifts were recorded in macroalgal assemblages, and common phaeophytes (*Padina* spp.) thrived despite decalcification in low pH environments (Johnson et al. 2012b). These observations have implications for the modelling of future impacts of ocean acidification on marine ecosystems.

The findings from this thesis have highlighted high tolerance to increasing atmospheric CO<sub>2</sub> for some benthic algal species and indicate that the primary productivity of many shallow water coastal habitats may be enhanced with increasing CO<sub>2</sub> emissions.

Promotion of benthic diatoms as CO<sub>2</sub> levels increase appears to be the main driver of this increased productivity. If this proves to be a widespread ecological response to increasing CO<sub>2</sub> levels, this is likely to have wide-ranging consequences: from local scale influences on the structure of overlying benthic communities, to effects on food web structure and larger scale biogeochemical cycles. The heterogeneous response of marine phototrophs to CO<sub>2</sub> has been highlighted by this research. This may be due to differences in environmental conditions across a variety of habitats and important taxa-

specific differences in carbon acquisition abilities. These non-uniform responses have the potential to perturb community composition and ecosystem function.

We are, however at the early stages of understanding the consequences of ocean acidification on marine benthic communities and discrepancies in the responses of algae to CO<sub>2</sub> enrichment across different studies still remain. The relative limitations and merits of *in situ* observations compared with laboratory manipulations have been previously discussed. Whilst laboratory and mesocosm experimentation has yielded many critical insights and is invaluable for determining the effects of a range of environmental variables and interactive stressors, it is difficult to extrapolate the findings to predict the effects on whole ecosystems. Volcanic vent systems are useful as they can reveal ecological responses to long term increases in CO<sub>2</sub> levels that retain natural pH variability (Fabricius et al. 2011; Kerrison et al. 2011) and acidify the water on scales large enough to integrate ecosystem processes such as competition and predation. Researchers have begun to reveal the underlying mechanisms that cause ecological shifts along these CO<sub>2</sub> gradients, such as the effects of high CO<sub>2</sub> on recruitment success (Cigliano et al. 2010), microbial colonisation (Lidbury et al. 2012; Johnson et al. 2012a) and herbivore-algal dynamics (Johnson et al. 2012b). It therefore appears that an effective way forward for future research is to combine both approaches to determine more explicitly the effects of ocean acidification (Barry et al. 2010; Wernberg et al. 2012). We also need to expand the use of *in situ* observations across different habitats and geographical regions to feed into global models predicting the effects of CO<sub>2</sub> emissions on marine ecosystems, as recently demonstrated by Johnson et al. (2012b).

There are a few future research recommendations, the experiments for which were beyond the scope and/or logistical means of this thesis but should form the focus of further research. Despite the inherent difficulties in controlling environmental variables along CO<sub>2</sub> gradients, recent research has shown that it can be possible for the potential interactive effects of temperature and CO<sub>2</sub> on marine biota to be observed *in situ* (Rodolfo-Metalpa et al. 2010, 2011). In these studies, observations of calcified organisms during a prolonged period of high seawater temperatures in areas of high CO<sub>2</sub> were compared with those made during cooler periods of the year. They also revealed that the adverse effects of global warming are exacerbated when seawater acidification coincides with high temperatures. Therefore future research in naturally high CO<sub>2</sub> areas should aim to undertake long term and seasonal observations in order to account for potential interactive effects of temperature and other environmental variables (such as light, nutrients, pollutants).

As an extension of the current research on microphytobenthic assemblages, it would be useful to adopt *in situ* optical techniques to measure the growth rate, biomass, photosynthetic activity and primary production of these assemblages in response to elevations in CO<sub>2</sub>. The use of *in situ* fluorometric techniques (e.g. Krompkamp & Forster 2003) may provide complementary data to support the conclusions discussed in this thesis; that high CO<sub>2</sub> stimulates the productivity of shallow water microalgal assemblages. For example, measurements of effective quantum efficiency and ETRs can be linearly correlated to O<sub>2</sub> production or carbon fixation. As these non-destructive methods are more rapid than destructive techniques such as chlorophyll extraction, they may yield important information on microphytobenthic dynamics over larger spatial and

temporal scales. Alternatively, net primary production estimates from  $^{14}\text{C}$  assimilation measurements may also be useful proxies for daily biomass production.

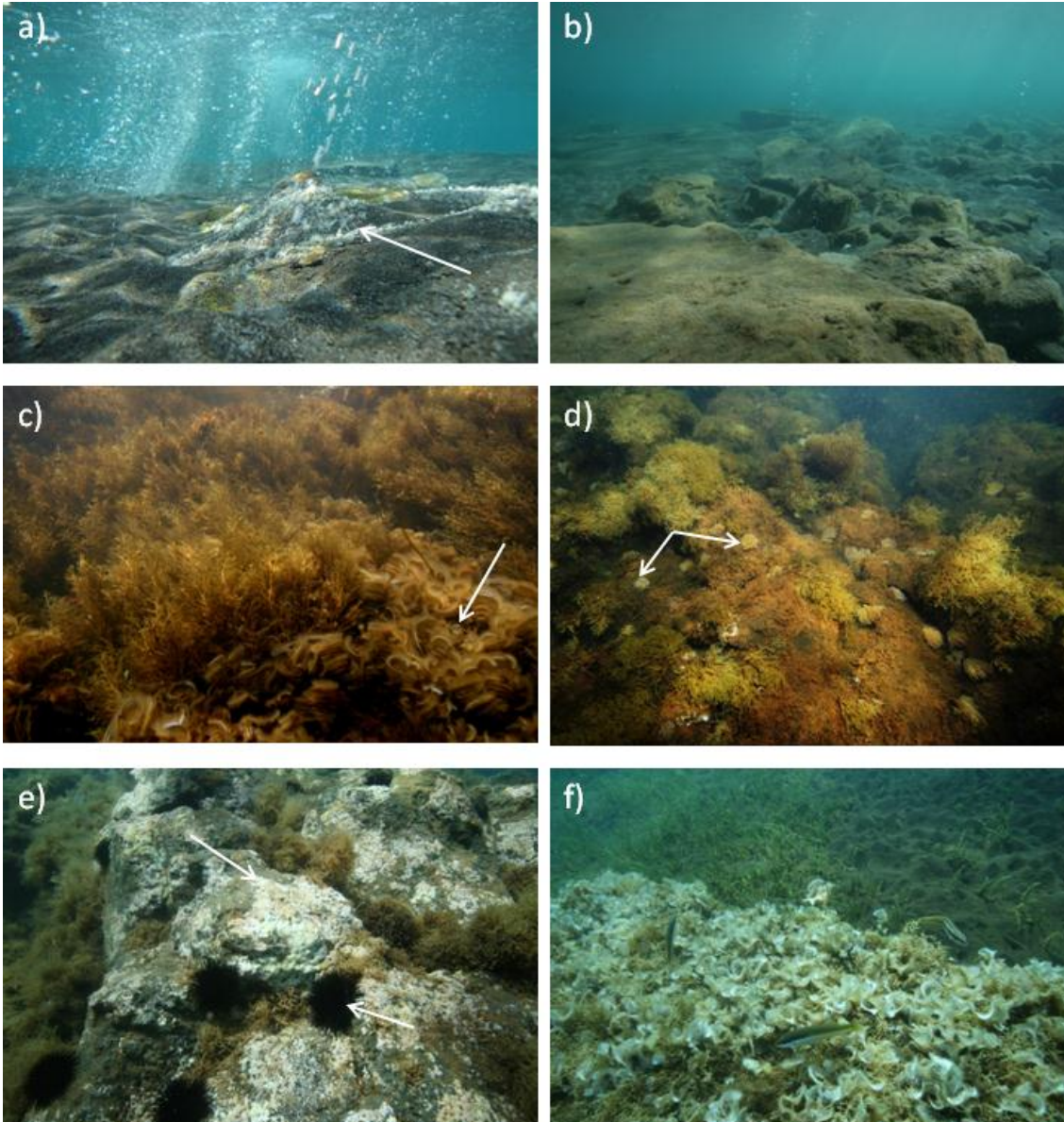
As previously stated, repeat investigations of endolithic algal assemblages and bioerosion rates along natural  $\text{CO}_2$  gradients, particularly in tropical regions, should form a research priority. This research could be developed further by more detailed analysis of endolithic bioerosion. For example, creating stained, petrographic thin sections of carbonate substrata to reveal the depth of endolithic filaments and using scanning electron microscopy (SEM) to quantify the surface area bioeroded by endoliths under different levels of  $p\text{CO}_2$ . This allows more accurate carbonate dissolution rates to be calculated, as demonstrated by Tribollet et al. (2009).

Additionally, a casting-embedding technique; impregnating bored substrata samples with epoxy resin, under vacuum (Golubic et al. 1970; Carreiro-Silva et al. 2005), followed by carbonate dissolution in hydrochloric acid, produces boring trace casts. These can then be examined with SEM to document the species composition and abundance of endolithic assemblages.

The response of marine organisms to long-term increases in atmospheric  $\text{CO}_2$  will depend on their ability to physiologically and morphologically adapt or acclimatise to expected changes in seawater chemistry. However, most empirical research on marine phytoplankton responses to ocean acidification to date has largely ignored the potential for contemporary evolution and research is limited by the short time scale of laboratory experiments. Marine microbes, with their large population sizes and fast division rates, are likely to evolve over a timeframe of decades and may be able to respond to environmental alterations through adaptive evolution (Collins 2012; Lohbeck et al. 2012). Volcanic  $\text{CO}_2$  vents can persist for millennia, therefore it may be possible to

investigate whether tolerant species have undergone genotypic adaptation to the long term CO<sub>2</sub> exposure. Utilising naturally high CO<sub>2</sub> areas in order to reveal the evolutionary responses of marine algae to ocean acidification should be a next step for ocean acidification research, in order to predict the adaptive abilities to the current rapid acidification of our oceans.

# **Appendices**



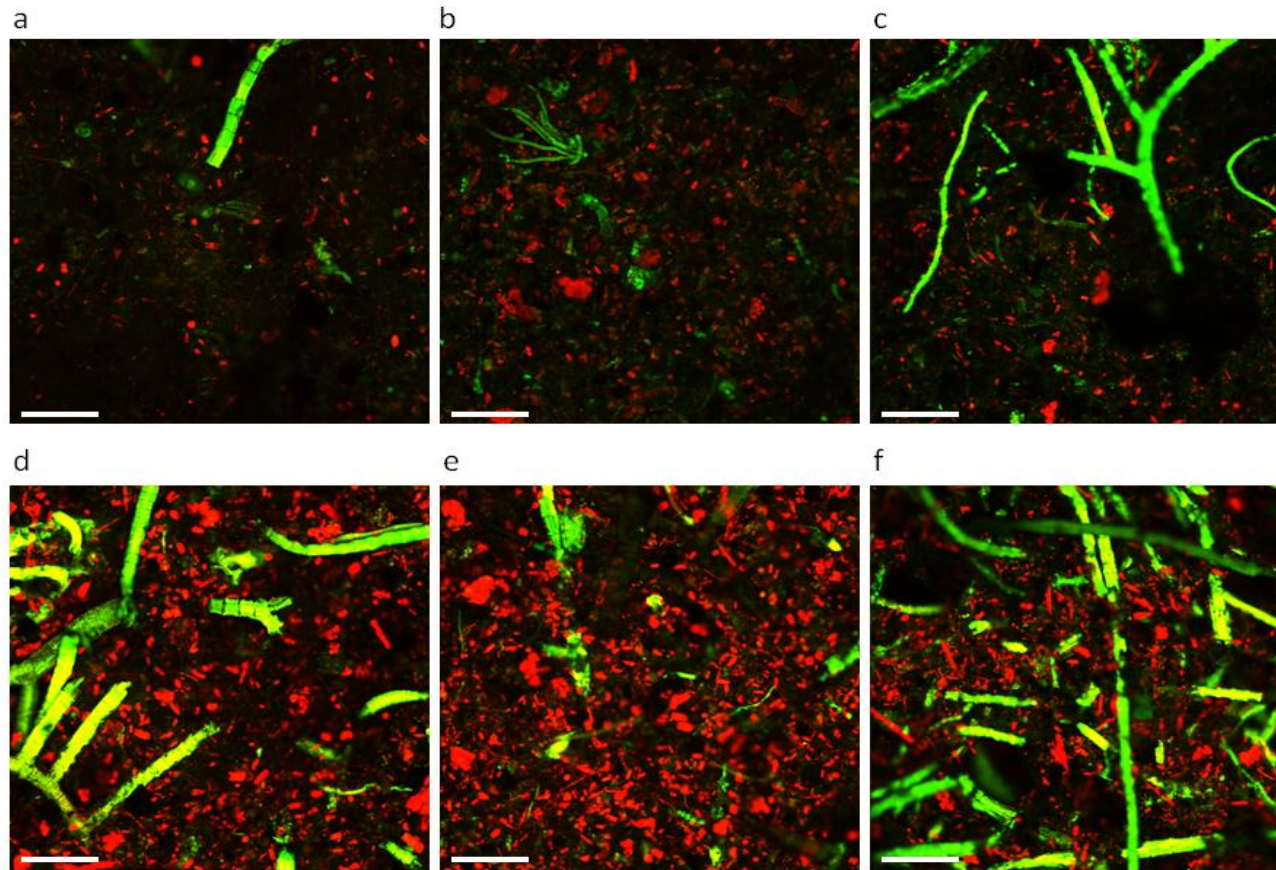
**Appendix I.** Photographs of benthic changes along the Vulcano CO<sub>2</sub> gradient.

a & b: The main vent source showing CO<sub>2</sub> seeping from the sea bed (a), only thermophillic biofilms (white coating, indicated by arrow) can tolerate the extremely low pH (~5.5) at these relatively barren sites (b).

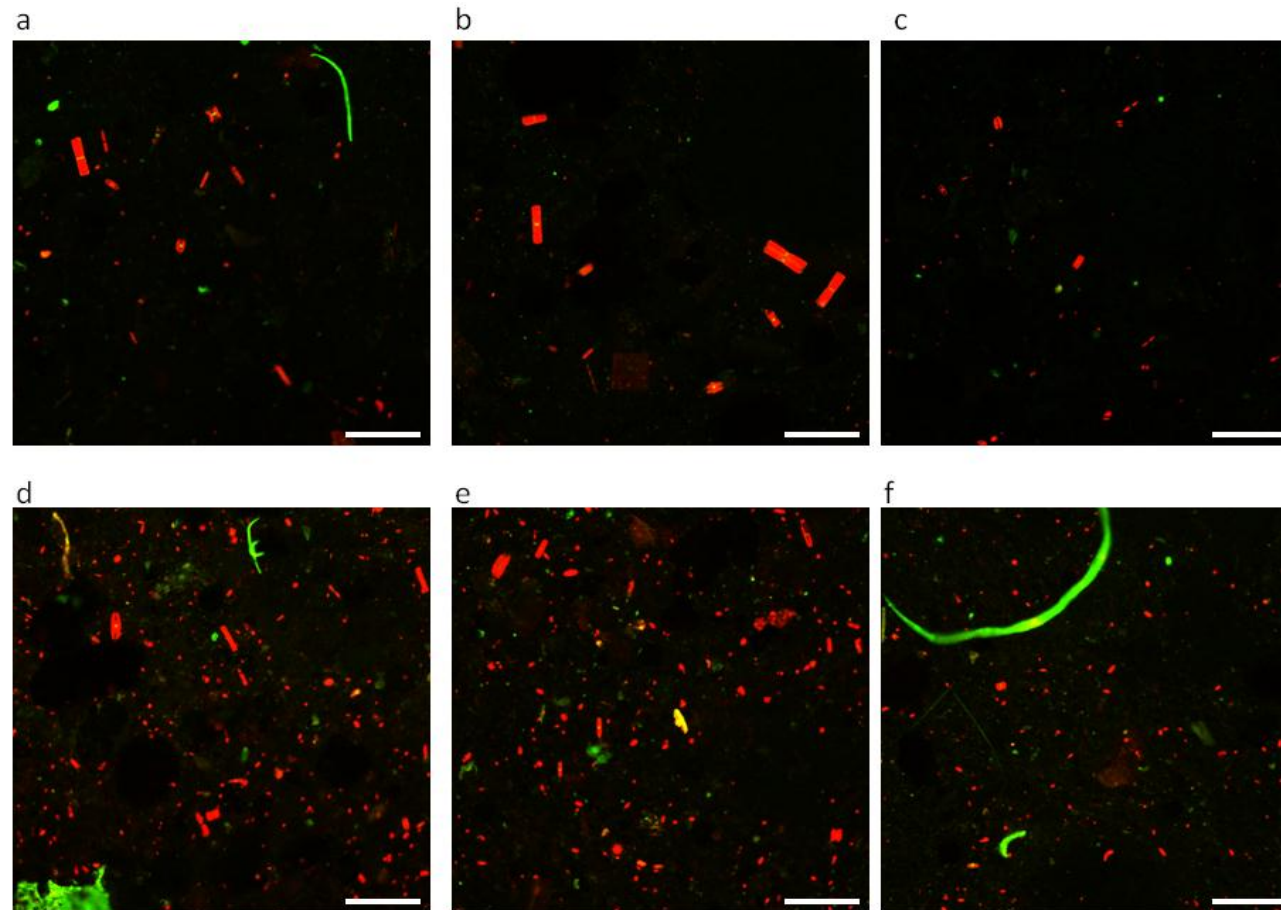
c & d: Areas elevated in CO<sub>2</sub> along the gradient (S2 & S3) showing the proliferation of phaeophytes, including decalcified *Padina pavonica* (c; Johnson et al. 2012b) and high abundances of sea anemones (d; Suggett et al. 2012). There is very little calcified biota at these stations.

e & f: Ambient conditions showing the abundance of calcified biota including crustose coralline algae (CCA), sea urchins (e) and calcified *P. pavonica* (f).






**Appendix II.** Epifluorescence images of biofilm material collected from subtidal rock surfaces, red = diatoms, green/yellow = algae containing phycobilins (both filamentous and coccoid cyanobacteria and filamentous rhodophyta); a-c = station R2, d-f = station S3 showing enhanced diatom abundance and proliferation of filamentous, phycobilin containing algae (predominately juvenile stages of rhodophyta), scale bar = 50µm.




**Appendix III.** Epifluorescence images of epipellic microalgae within sandy subtidal sediments, red = diatoms, green/yellow = cyanobacteria; a-c = R4, d-f = S3 showing increases in diatoms and cyanobacteria abundance, scale bar = 50 µm.

**Appendix IV.** Summary of data derived from experimental calcium carbonate substrata following a ca. 7 month installation period at stations R4, S1-S3. Data values represent means, standard error values given in brackets.

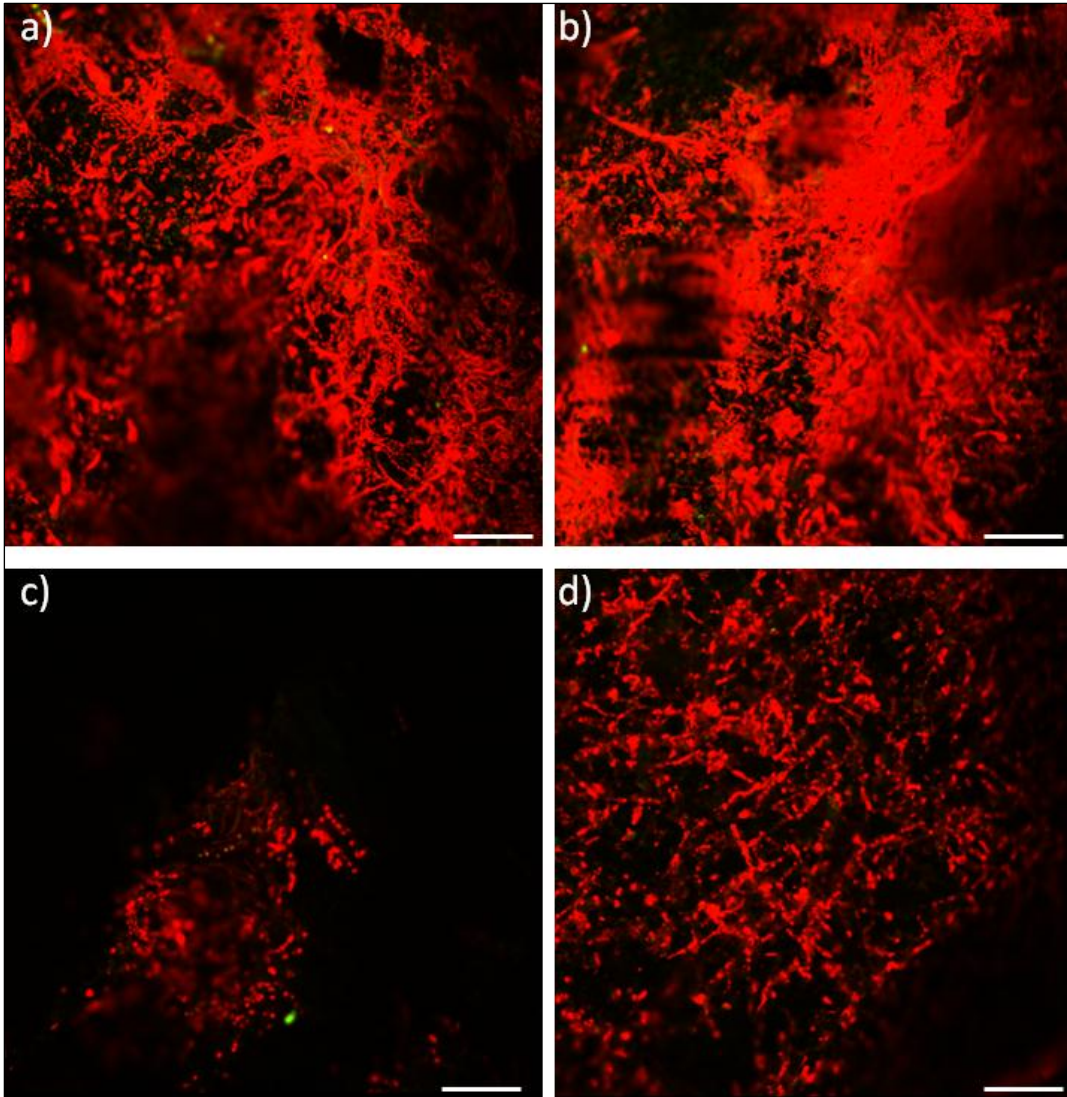
Station and exp base		CaCO <sub>3</sub> Blocks					Limpets ( <i>Patella vulgata</i> )					
		no. replicates survived after 7 mo.	% Reduction in cubic volume	Endolithic Chl <i>a</i> (µgcm <sup>-2</sup> )	Epi-fluorescence		no. replicates survived after 7 mo.	% Reduction in weight	% Reduction in shell thickness	Endolithic Chl <i>a</i> (µgcm <sup>-2</sup> )	Epi-fluorescence	
					Cyano-bacteria (% cover)	Chloro-phyta (% cover)					Cyano-bacteria (% cover)	Chlorophyta (% cover)
R4		X					X					
S1	A	3	16.3 ± 6.0	0.64 ± 0.2	1.25 ± 0.2	19.9 ± 3.5	n/a	n/a	n/a	n/a	n/a	n/a
	B	4	12.0 ± 4.3	0.37 ± 0.1	-		7	2.7 ± 0.5	12.7 ± 4.6	1.12 ± 0.2	-	-
S2	A	5	19.7 ± 2.2	0.82 ± 0.2	-		n/a	n/a	n/a	n/a	n/a	n/a
	B	5	21.3 ± 3.7	1.67 ± 0.5	0.88 ± 0.1	29.4 ± 3.2	8	9.6 ± 1.2	22.4 ± 4.4	1.38 ± 0.2	1.68 ± 0.1	37.9 ± 2.5
	C	11	21.0 ± 2.7	2.73 ± 0.5	-		n/a	n/a	n/a	n/a	n/a	n/a
S3		X					X					

 = experimental base dislodged from original placement and upturned (length of time substrata exposed to reduction in light unknown)

 = experimental base exposed to ca. 56% light reduction throughout 7 mo. experimental period

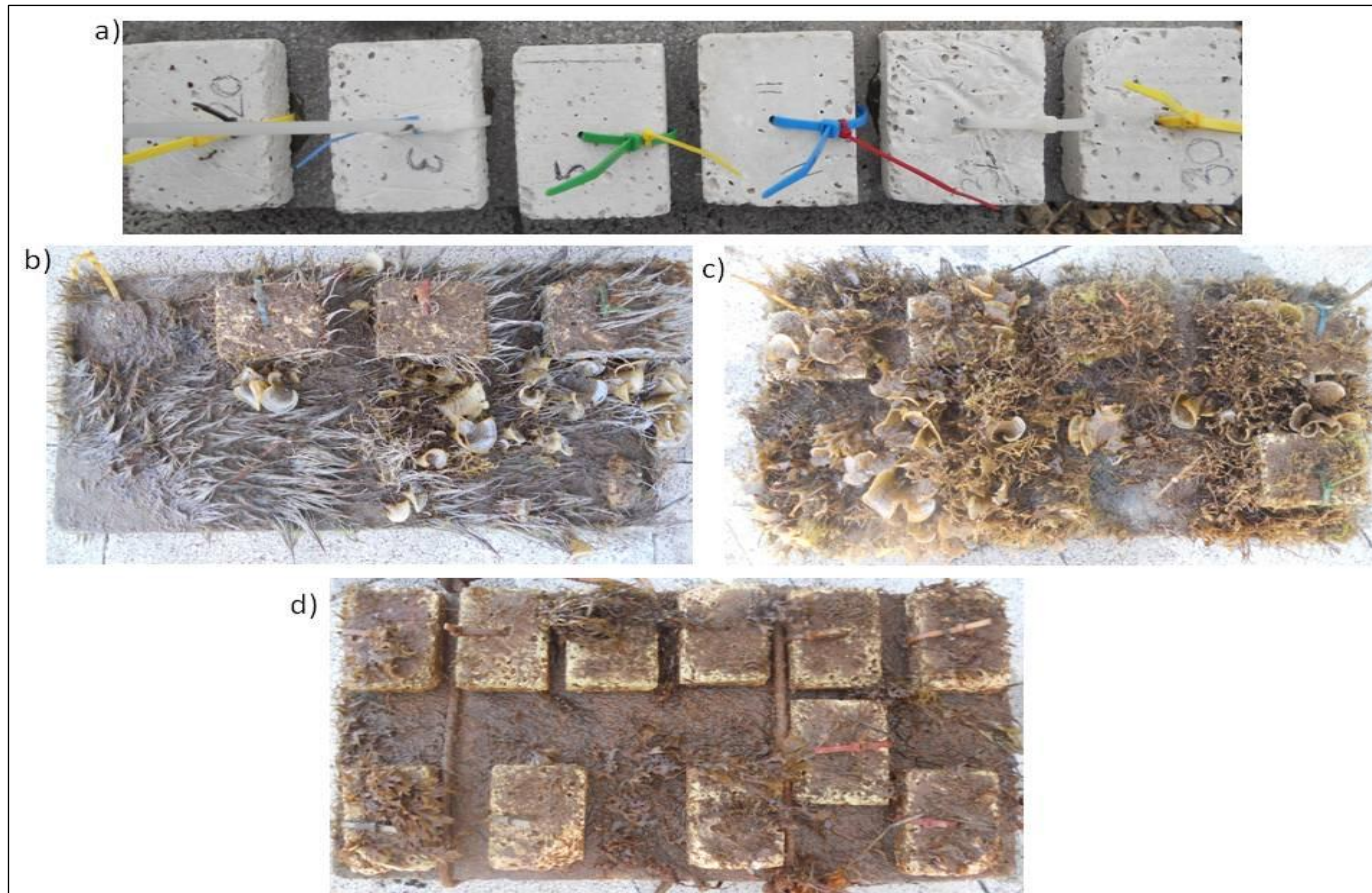
**X** = experimental bases absent after ca. 7 month transplantation period

**-** = not tested



**Appendix V.** Epi-fluorescence images of endolithic microalgae found in the upper surface layer of *P. caerulea* shells from stations R2 (a & b) and S3 (c & d). Red = chlorophyta green/yellow = cyanobacteria, scale bar = 50  $\mu\text{m}$ .





**Appendix VI.** Photographs of the cement bases containing the experimental  $\text{CaCO}_3$  substrates; a) aragonite blocks pre-submersion, coloured cable ties allowed identification of individual blocks for size measurements before and after installation, b-d) after the ca. 7 month installation period at station S1 (b) and S2 (c & d) with algal overgrowth. Bases 'b' & 'c' were exposed to ambient light levels whilst base 'd' was installed under pier pilings where light intensity was reduced by  $\sim 54\%$  .

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